Title Cryopreservation Of Zebrafish (Danio Rerio) Blastomeres Using Controlled Slow Cooling

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ABSTRACT

Cryopreservation of aquatic species has been widely studied especially zebrafish gametes, embryos and larvae. Cryopreservation of blastomeres has the advantage of preserving both paternal and maternal genetic information.

The research work presented in this thesis investigated the toxicity of cryoprotectants to 75% epiboly stage blastomeres of zebrafish (*Danio rerio*) before cryopreservation. DMSO was found to be the least toxic cryoprotectant for blastomeres, after 30 min incubated in PBS at room temperature, with 97.8% survival. Cryopreservation of zebrafish blastomeres was carried out using controlled slow cooling. In addition to cryoprotectant, the cryoprotective properties of other compounds were also investigated including $NaHCO_3$, coffee and honey, and their solutions were used as cryopreservation media. Comparison of blastomere survival in different media after freezing showed honey to be the most effective, with 98.1% survival immediately after freeze thawing. Comparison of blastomeres cryopreserved in honey and DMSO, after 60 min incubation in PBS at room temperature following freeze-thawing and cryoprotectant removal, showed honey to be the more effective cryoprotectant for controlled slow cooling of zebrafish blastomeres with 97.2% survival. Results from the present study showed honey to have properties that protect the blastomere from freezing injury.

TO MY BELOVED FAMILY

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CHAPTER 1

INTRODUCTION

1.1 PRINCIPLES OF CRYOBIOLOGY

Cryobiology studies the effect of low temperatures on biological systems after freezing and thawing in order to achieve long-term sustained viability. This growing branch of biology is intended to preserve the structural and functional integrity of the cells, tissue and organs after freezing. Cryobiology's challenge is to preserve the living system at cryogenic temperatures in liquid nitrogen for unlimited periods of time. Gametes and embryos cryopreservation of many species has been achieved, improved and simplified, during the last three decades, so they can now be stored for long periods of time, and develop normally from the low temperature storage (Devireddy et al., 2004).

New techniques are being tested and improved to make cryopreservation a safer and harmless practice to preserve life. Cryobiological discoveries and techniques are playing an important part in human infertility treatment, organ and tissue transplant and preservation of biodiversity.

Many of the cryobiological techniques have existed for long time and they are now considered commonplace biological and medical procedures, while others are still regarded as experimental to biology and medical world. Such techniques include cryosurgery, cryotransport, gamete and embryo preservation (cryopreservation), tissue and blood preservation, and tissue transplantation.

In order to achieve higher viability of cells or tissues after freezing, an appreciation of the chemical events that occur during freezing and thawing is required. It is important to plan the appropriate strategies to consider the significance of other key steps such as tissue acquisition, thawing and post-thaw processing of the cell and tissue, as every step can have harmful effects.

1.1.1 CHILLING INJURY

There are two types of chilling injury: direct chilling injury and indirect chilling injury.

Direct chilling injury, also known, as cold shock injury is dependant on the cooling rate. The injury is increased when the incubation period at low temperature is extended. Membrane permeability diminishes after rapid cooling, though it may be reversed in some cases by re-warming. The main cause for direct chilling injury is the thermo tropic behaviour (phase transitions) of the lipids in the membrane (Morris, 1987b). Lipids in cell membranes undergo a liquid-to-gel phase transition in a range between 0°C and 20°C, the temperature range of maximum chilling injury. The nature of this injury is not completely understood, but could be related to this phase transitions, altering the properties of the membrane and its proteins and enzymes (Cossins, 1983).

Indirect chilling injury is independent of the rate of cooling; it becomes apparent after a long period of exposure to temperatures at around 0°C (Morris and Clarke, 1987a). Indirect chilling injury increases with exposure time at critical temperatures, but it may appear earlier and can occur within minutes specially when cooling mammalian oocytes and embryos (Leibo et al., 1996). Rapid cooling through the critical temperature range (to minimize the time spent in the phase transition region) can be a means to reduce chilling injury (Mazur et al., 1992b).

Low temperatures affect the function and structure of proteins as enzyme activity is decreased and proteins suffer denaturation. The disarray of metabolic and enzymatic processes is even more harmful in fast growing embryos (Mazur et al., 1992a) as those of the fruit fly (*Drosophila Melanogaster)* and zebrafish (*Danio rerio*). Chilling injury has also been reduced in fish & pig embryos by removing lipids (yolk) (Drobnis et al., 1989).

1.1.2 Freezing Injury

When water freezes inside the cell, it is generally considered fatal (Mazur, 2004), if freezing occurs around the cell, it induces a risk of damage as the increased osmotic pressure of the external solution due to the ice, causes water to flow outward, this process may produce the rupture of the plasma membrane. The degree of cell injury varies depending on several factors such as the cooling rate, the end temperature and the thawing rate, etc.

The most important impact of reduced temperatures on any system is the reduction of molecular motion, which causes the rate of biological or chemical reactions to slow down (Grout and Morris, 1987). In addition, the physical properties of water are very sensitive to temperature, and these properties show remarkable changes as temperature changes, especially to temperatures below 10°C (Morris and Clarke, 1981). The transition from the fluid to the gel phase reduces the activity of membrane bound enzymes and induces bunch formation of integral membrane proteins (Hazel, 1995).

Some cell membranes also are very impermeable to large molecules even when they are non-polar or neutral species. Such large molecules need specific transporters to cross the cell membrane and this type of transport is termed as facilitated or carrier mediated passive transport. Glucose transport is a typical example of facilitated diffusion (Becker et al. 1996). Therefore, the rate of solute entry is directly proportional to the quantity of transporters that is occupied with solute. The study of the transport mechanisms at low temperatures may provide essential information thus some research is needed to elucidate the understanding of interchange cellular membrane permeability temperature.

For most cells, a major factor that needs to be considered when freezing to sub-zero temperatures is the rate at which they are cooled, controlled rate cooling is required for their optimal survival and viability (Mazur, 2004).

In order to achieve a successful cryopreservation using controlled slow cooling, an optimal controlled cooling rate is to be found. During optimal cooling, between -5 and -15 degrees, external ice will form, spontaneously or by 'seeding', the cell then would go through dehydration (intracellular water flows out due to osmotic pressure)

to increase the solute concentration and keep the chemical potential in balance. Cells frozen with little or no intracellular water will survive after freeze-thawing.

A reduction in temperature can affect significantly the physical state of the cell membrane, since it can change from an ordered state (crystalline gel) to a fluid state (liquid crystalline) and vice versa. As the temperature drops the fluidity of the membrane changes causing the fatty acid saturation to decrease, while maintaining the basic interactions with the membrane proteins necessary to keep the membrane working. One key characteristic of membrane adaptation is the 'increased efficiency of nutrient uptake' at low temperatures.

Most cells have the ability to alter their membrane fatty acid composition as temperature changes in order to keep their membrane at nearly the same fluidity despite the temperature changes (Becker et al., 1996). Organisms possess several protection mechanisms against temperature stresses such as production of cold and heat shock proteins and altering membrane fatty acid composition in a process known as "homeoviscous adaptation".

Cold shock proteins help maintain the cells' original structure and functioning by halting "protein synthesis until the cells have acclimated to their new environment.' Ice nucleation proteins mimic the behaviour of ice crystal, which helps delay the internal damage to the cell by releasing a small amount of heat. Also, research has shown that a special protein exists which can inhibit ice nucleation and antifreeze activity. However, these proteins are not helpful for long-term survival of the cell since the 'thermodynamically unstable crystals have a tendency to reform into large, damaging structures'.

Rapid responses by cells in vitro during a freeze thaw cycle include cellular osmotic behaviour. At different low temperatures the cells show changing osmotic responses. These responses can affect significantly the chances of survival of the cell during a freeze thaw cycle. At low cooling rates the cells are able to maintain a chemical equilibrium by losing water at a fast enough rate. As the rate of fall in temperature increases, the rate of ice formation is greater than the rate of water loss, making the inside of the cell to become supercooled (Ponder, et al, 2004).

1.1.3 CRYOPROTECTANTS

Cryoprotectants are chemical agents that assist the cell freezing process and recovery after freezing. They are essential for the cryopreservation of almost all biological systems. Their molecular structure determines their permeating condition, thus they can be (a) permeating cryoprotectants, e.g. methanol, dimethyl sulfoxide (DMSO), glycerol and propylene glycol (PG) when their molecular weight is small enough to penetrate the cell membrane or (b) non-permeating cryoprotectants, e.g. hydroxyethyl starch, polyvynil, pyrrolidone, and a variety of sugars when molecular weight is high and cannot go into cells. Cryoprotectants of each group play different roles during cooling and thawing (Zhang et al., 2007).

Permeating cryoprotectants produce a substantial freezing point depression in addition to that due to any electrolytes present within the system, eventually leading to a ternary (cryoprotectants-salt-water) eutectic point at a low temperature (Shepard et al., 1976), as the increasing salt content of remaining liquid is the main cause of so-called "solution effects" (Mazur, 1965) cryoprotective properties is called also osmotic buffering (Zhang et al., 2007).

The non-permeating cryoprotectants play a cryoprotective effect during dehydration of cells prior to cooling, this reduced ice crystal formation during freezing. Some high molecular weight (<50,000) cryoprotectants protect cells during freezing and thawing by changing ice crystal formation to a harmless size and shape (Mazur, 1965). Even as huge alteration of cellular morphology can be protect by cryoprotective agents, a diversity of cryoprotectants can themselves be harmful to cells when used in high concentrations, (Fahy, 1986; Arnaud and Pegg, 1990; Pegg and Arnaud, 1988).

Naturally occurring non-permeating cryoprotectants in high concentrations are sometimes preferred over permeating solutions. Some widely used non-permeating cryoprotectants are egg yolk and milk proteins, which contain proteins such as: 'amides; synthetic polymers such as Ficoll, polyethylene glycol, polyvinylpyrrolidone or methyl cellulose; and algae-derived polysaccharides such as agarose and alginate' (Sher et al. 2007).

These cryoprotectants protect the cells during cryopreservation by stabilizing the cell membrane. These compounds belong to membrane- stabilizing group formed by 'egg yolks, milk, lipids, sugars, amino acids, glycerol, 1.2- propanediol, polyvinylpyrroli done, and bovine serum (BSA)' (Santoro et al., 1999; De Leeuw, et al., 1993). In addition, these compounds do not penetrate the cell because they have large sugar molecules, such as raffinose, sucrose trehalose and Ficoll. (Trounson, et al., 1999).

All cryoprotective agents are highly soluble in water. Cryoprotectants are likely to form stable hydrogen bonds with water molecules, the permeating cryoprotectants decrease the freezing point of the solution as they tend to produce, with the present of electrolytes (salt) and the water, a ternary low temperature eutectic point, allowing them to reach lower temperatures before freezing, to help protect the cell from freezing injury (Shepard et al., 1976). The second key property of these agents is that they should be non-toxic to the cells that they are supposed protect, the toxicity of these agents is a key limiting factor in cryobiology, especially when used in high concentrations (Fahy, 1986; Fahy et al., 1990). Although from a theoretical point of view they should be able to fully suppress all known types of freezing injury, they do not usually permit 100% survival after freezing and thawing. It seems that not only does the toxicity prevent the use of fully protective levels of cryoprotectants; it has also been considered to cause a level of injury itself, beyond the cryoinjury due to classical reasons. If a better control of toxicity is achieved it will in due course lead to significant progress in cryopreservation (Plachinta et al., 2004).

Some cryoprotectants have been proven to be effective through many years of experimentation including DMSO, glycerol, ethylene glycol, methanol, propylene glycol, glucose and sucrose.

The sugars in the freezing solutions, usually sucrose, serve to essentially dehydrate the embryos before they are exposed to cooling temperatures. This protection is vitally important to the embryo, since an embryo is comprised mostly of water and water-soluble salts and macromolecules. If water is not removed from the embryo before cooling, it can form ice crystals inside the cells that can puncture through delicate cell membranes like a sword. Cell membranes are protein and lipid bilayers that surround the cell, maintaining its integrity and physiological functions. If ice

crystals slice them, the membranes may not be able to keep the cells intact, causing severe cell damage. When the cell damage is extensive, an embryo may not be able to recover and will not survive the freezing and thawing process (Gleason, 2002).

The two disaccharides -sugars composed of two simple sugars- that most protect proteins & cell membranes against chilling, freezing & dehydration are sucrose (fructose, glucose) and trehalose (glucose, glucose). Sucrose is the most common sugar found in freezing-tolerant plant, which can increase their sucrose levels, ten-fold in response to low temperature. Sugars are more often used as cryoprotectants against freezing and chilling injury rather than for vitrification, with the disaccharide sucrose being more effective than the monosaccharide glucose (Carpenter et al., 1988, Crowe et al., 1988).

1.1.4 Approaches used in Cryopreservation

The challenge to a living system during freezing is not their survival once it has reached the storage stage at very low temperatures, usually below -180°C, it is rather the lethality of the transitional zone of temperatures from -1 to -60°C that a cell must pass through during the cooling procedure and again during thawing. There are three important steps that need to be taken before freezing: a) assess the possible chilling sensitivity of the cells to be frozen; b) study membrane permeability by determining the membrane permeability coefficients or the radio labelled CPT's, and c) study cryoprotectant toxicity to cells by evaluating and determining the type and concentration of cryoprotectant (Plachinta et al., 2004; Franks, 1981).

To avoid the possible risk of cryoinjury, there are two main approaches used in cryopreservation: Controlled slow cooling and vitrification.

1.1.4.1 Controlled Slow Cooling

During cryopreservation, if the cooling rate is too slow, the cell can be damaged by extreme dehydration and shrinkage whereas if the rate it is too fast, the cell will not

have enough time to dehydrate increasing its chances of producing an ice nucleation with a fatal consequence, as internal ice formation (IIF) may occur (Grout, 1991). When cells are cooled at a slow rate there are two different ways they could be damaged, one is due to the solute toxicity that apparently results from cell's balance alterations that provoke biochemical and biophysical distress (Lovelock, 1953b; Mazur et al., 1972). The second is thought to be as resulting from the shrinkage of the cell when exposed to hypertonic solutions (Lovelock, 1953a; Meryman, 1970, 1974).

If cells are immersed in a solution of a penetrating cryoprotectant before freezing, their viability might be enhanced significantly, provided the toxicity of the cryoprotectant is reduced to a minimum, after finding the appropriate molar concentration and the optimum cooling rate.

In order to improve the viability of the cells when using controlled slow freezing, it is required to follow certain steps:

a. Collect cells and assess quality.

b. Equilibrate cells in cryoprotectant.

c. Freeze cells using controlled slow cooling to –80°C.

d. Plunge and low temperature storage at –196°C.

e. Warm and thaw cells using controlled conditions.

f. Remove cryoprotectant, return cells to normal physiological conditions.

1.1.4.2 VITRIFICATION

Temperature drop causes the slowing of molecular motion, and when a critical temperature is attained there is virtually no energy for these molecules to move. At this temperature, water loses its fluidity and becomes a glass and is said to have vitrified, remaining basically as a liquid in which molecular motion is clogged, but with the mechanical properties of a solid (Farlane, 1987; Farlane et al., 1992).

It is always best to avoid ice when possible, ice formation is a two-step process involving first the formation of a nucleus (nucleation) and second the growth of an ice crystal from the nucleus, and given that physical damage is produced to the connective tissues found in organs during ice formation, tearing down capillaries and the fragile relationships between cells, rendering them useless, vitrification has been considered a possible solution, in many cases even better than controlled slow cooling.

During vitrification water increases its viscosity level during the cooling process, ending eventually in a fluid without inner motions just as it is in a solid crystal, remaining stable over time, without the molecular rearrangements produced through crystallization that cause massive damage to cell structure and stability (Meryman, 1974).

Pure water normally freezes, but water solutions containing concentrations of soluble chemicals can allow water to vitrify. Some of these chemicals are agents that have been customarily used to protect cells from freezing and thawing damage, (Rassmusen and Luyet, 1970).

When the concentration of these cryoprotectants (CPAs) is high enough, it would allow vitrification, preventing ice formation and/or freezing damage by increased viscosity, by hydrogen-bonding with water molecules, by dilution of electrolytes and/or by colligative interference and locking all of the cell's molecular elements in the process, leaving them unable to change eventually.

Vitrification prevents damage connected to intracellular ice formation and intracellular ice recrystallization during rapid freezing and thawing, cellular dehydration and shrinkage, and exposure to elevated intracellular and extracellular solute concentrations that occur during slow freezing, all of which can produce irreversible harmful effects (Fahy et al., 1984).

Although vitrification is a promising method for cryopreserving biological materials in order to obtain an adequate medium for vitrification, special characteristics need to be considered. (1) cryoprotectants used need to have a high permeable concentration and the permeable and non-permeable agents need to be tested for toxicity levels; (2) the process needs to incorporate high molecular weight compounds, such as sucrose

solutions. In some cases, the addition of cryoprotectant solutions is not enough to guarantee a successful vitrification of embryos, as shown in studies by Cabrita et al., (2003) and Janik et al., (2000).

1.2 APPLICATIONS OF CRYOBIOLOGY

Cryopreservation has been used for gametes, embryos and organ preservation, long-term storage of cells and tissues. Applications of Cryobiology also include cryosurgery, insects and plants lyophilization (freeze-drying) etc.

1.2.1 APPLICATIONS IN BIOMEDICINE

The metabolism in living cells dramatically diminishes at low temperatures, cooling at low temperatures permits the long-term preservation of living cells and tissues for either medical research or medical industrial applications such as organ transplant, blood transfusion, bone marrow transplantation, artificial insemination, in vitro fertilization, tissue storage, etc.

Human spermatozoa was first successfully frozen with glycerol reaching a 67% survival of motility by Bunge and Sherman in 1953, after being frozen in dry ice they produced pregnancies in three woman by artificial insemination (Bunge and Sherman, 1953). Sherman found that deep subzero storage temperatures were essential to ensure a substantial survival and viability assessed through spermatozoa motility. However, cryopreserved spermatozoa were three times less successful into achieving pregnancy than its fresh equivalent (Richter et al., 1984).

Preservation techniques in human embryos came after being tried in animals, as happened with many other cryobiology developments. A moderately successful protocol using slow cooling rate, DMSO and rapid warming became a standard (Gelety and Surrey, 1993). Later 1,2 propanediol, containing sucrose as an osmotic buffer, proved to be a better CPA, especially with single-cell embryos, provided slow cooling should be stopped at –30°C and rapidly warmed (Testart et al., 1986). In most

of these procedures the adding and removal of cryoprotectant needs to be done in meticulous steps to prevent osmotic stress.

Vitrification has been achieved with clinical blastocyst-stage human embryos (Choi et al., 2000) and vitrification using ethylene glycol and DMSO ended with a successful healthy pregnancy (Yokota et al., 2000).

The application of ICSI (intracytoplasmic sperm injection) to oocytes has been a foremost advance that has resulted in an improvement of the chances of fathering a child for infertile males. Slow cooling protocols in which the sodium chloride has been replaced with choline to try to prevent salt damage are being tested and evaluated (Quintans et al., 2002), but there is still a lot of work to be done to improve viability.

In 1983, Trounson et al. reported the first successful replacement of a frozen-thawed embryo in Australia. This advance in science was an important contribution to the study of cryopreservation. The incidence of blastomere fusion after cryopreservation of early human embryos (blastocyst stage) was investigated (Balakier et al., 2000). The use of freezing and thawing has been shown to induce changes in the chromosome of human embryos by Selick et al., (1995) as well as reduce the embryo's "viability and the number of embryos available for uterine transfers" (Selick et al., 1995). It was reported by Wood (1997) that survivals of embryos after exposure to freezing and thawing were 17-70% and in some cases undergo degeneration blastomer. As a result, cryopreservation may be regarded as a damaging process that can also induce "cracks in the zona peludica, or injuries to the cell membranes and intacellular components (Ng *et al.* 1988, Dumoulin *et al.* 1994, (Mozdarani and Moradi, 2007).

The present observations may also suggest that early human embryos are more susceptible to cryodamage and blastomere fusion when compared with older, more advanced embryos. It is likely that the properties of cell membranes, for instance fluidity, are changing during embryo development, and perhaps for this reason more fusion has been observed freeze-thawed embryos. Studies (Trounson 1984; Balakier et al., 2000) it showed that freezing and thawing is responsible for blastomere fusion, and this may occur regardless of the type of cryoprotectants used. Research on cryopreservation of mammalian embryos has focused on major variables influencing

the ability of an embryo to survive a freeze-thaw process. Emphasis has been placed on optimal techniques for successful embryo storage.

1.2.1.1 CRYOSURGERY

Cryosurgery is a surgical technique that uses freezing to destroy malignant tissues, while minimizing injury to the healthy surrounding ones. Cryosurgery is being used on cancers of liver, skin, prostate and breast (Hoffman and Bischof, 2004). It has been considered a part of cryobiology even though the object of cryosurgery is not to preserve but rather to kill cells. Cryosurgery essentially involves localised rapid cooling to lethal temperatures followed by relatively slow warming and is used to eliminate unwanted cells such as tumour cells.

Cryosurgery works by exposing patient's tissue to subzero or even deep subzero temperatures, causing water inside cells to freeze. Intracellular freezing is usually lethal, and its lethality is enhanced by slow warming. The destructive process of freezing is yet being discussed, as there are several theories regarding the way and the mechanisms by which freezing destroys tissue. The first and oldest is that of direct cellular injury caused by protein damage as the cell dehydrates through freezing (Lovelock, 1953a, 1953b). Another theory is that of a destructive immunological response triggered by the initial damage to tissue produced by the freezing insult (Ablin, 1995). The third hypothesis is the one caused by vascular injury at a capillary level restraining the tissue supply rendering it ischemic and eventually necrotic (Daum et al., 1987).

Striking reductions in cancer perseverance have been achieved through cryosurgery when compared to conventional surgery alone, the reason for this important advantage is the cryoimmunologic damage-provoked effect that results when target cell antigens are explicitly released to the immune surveillance mechanisms, stimulating the immune system (known now as "cryoimmunological response") (Ablin et al., 1974).

1.2.2 APPLICATIONS IN HUSBANDRY AND CONSERVATION

Conservation is the part of cryobiology that is engaged with the procedures to suitably freeze biological systems to deep low temperatures that will slow and virtually stop their metabolic activity permitting a long-term storage. Hypothermia and freezing is not new for many species of microorganisms, plants, insects, and reptiles that survive every winter in extreme cold weather zones using a range of strategies to avoid or stand freezing within their tissues. There is plenty to be learnt from these natural systems (Schmidt, 1982).

1.2.2.1 HUSBANDRY

The research and development that recently devised biotechnologies have conveyed to the assisted reproduction, mainly targeted at breeding of cattle, have also pressed on the advance of cryobiology. Techniques such as '*in vitro*' maturation and fertilization of oocytes, and genetic engineering intended to alter the genome of animals, have transformed the field of animal reproduction entirely and it has required the cryopreservation of gametes, embryos and other cells and tissues (Massip et al., 2004).

The collaboration of Walton and Hammond (Hammond, 1930; Walton, 1930) produced the first insights on artificial insemination. Then there was a huge step forward given by the fortuitous discovery of the cryoprotecting effect of glycerol on fowl spermatozoa, by Polge (1949) adding later similar findings and results using ethylene glycol and propylene glycol (Polge, 1968).

Cryopreserved mammalian semen is generally acknowledged to have an impaired fertility by comparison with fresh semen, the possible effects of cryopreservation on the role of spermatozoa in the early stages of embryogenesis are considered. The reasons for the loss of fertility are various, e.g., cold shock susceptibility, cooling rate, diluent's composition and osmotic stress (Watson, 2000).

Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function have been studied (Russell et al, 1997;

Curry, 2000; Thurston et al., 2003). Research on the cooling and cryopreservation of mammalian spermatozoa is reviewed. The functional state of frozen and thawed spermatozoa is examined on the basis of published results of structural and functional tests of sperm competence, the importance of this to the utilization of cryopreserved spermatozoa is examined, and proposals are made for new avenues of research to overcome these problems (Watson, 1995).

In 1972 Mammalian embryos were successfully frozen and thawed for the first time. Subsequent to the first reports of successful cryopreservation of mouse embryos (Whittingham et al., 1972; Wilmut, 1972), live offspring have been produced from frozen embryos in many other mammalian species including human and cattle.

Experiments on the low temperature preservation of cow embryos (Wilmut et al, 1973) led to the development of embryo transfer technology in domestic species. Other Studies evaluated the effect of freezing-thawing procedures on the viability of sheep embryos cryopreserved at various developmental stages (Garcia-Garcia et al., 2006). Results showed early sheep embryos are very sensitive to freezing per se and the survival rates following conventional freezing improve as embryo developmental stage progresses.

1.2.2.2 CONSERVATION OF CELLS AND TISSUES

The techniques developed through all the related disciplines close to cryobiology are making possible the creation of genetic banks for conserving biodiversity for future research. A genome resource bank is a systematic collection of biological material containing somatic tissues, cultured cell lines, DNA, and germplasm that may be used for breeding or scientific research (Holt et al, 1996).

As for the cryopreservation of isolated cells, the only concern is the response of individual cells that are usually suspended in an extracellular solution. Since there is only one cell type, the reaction of the rest should be identical, except for a slight variation within a population like cells of different size, or at different stages of development, etc. In the case of tissues (i.e. an association of cells with a similar structure and function), circumstances are far more complex, as there are different cell

types that may respond quite differently to identical freezing and thawing conditions and the presence of extracellular structure can affect the freezing process as well as the cellular response. Tissues are three dimensional, so the ways heat and mass move throughout its structure are extremely important factors (Muldrew et al., 2004).

1.3 CURRENT STATUS OF AQUATIC SPECIES CRYOPRESERVATION

Fish embryos are difficult to cryopreserve, mainly because of their large size resulting in a low surface area/volume ratio, low membrane permeability and a bulky egg-yolk. However, oocytes have a simpler structure and higher membrane permeability. It has been found that the permeability of immature oocytes to water and cryoprotectants in the medaka fish (*Oryzias latipes*), is outstandingly higher than that of mature oocytes (Valdez et al., 2005). These attribute could make cryopreservation easier to achieve. Cryopreservation of genetic materials of aquatic species plays an important role in aquaculture, conservation and biomedicine.

Gene banks are important for maintaining genetic variety of fish populations that are endangered and to protect them from extinction. More than 65% of the European fish species are threatened (Kirchofer, 1996) or endangered making cryopreservation a very important tool to increase the representation of genetically valuable animals, to avoid genetic losses through disease, catastrophe, and making possible the transfer of stocks between hatcheries.

1.3.1 SPERM

Cryopreservation allows the collection of sperm of the highest quality, preserving semen of selected strains or genetically improved fish populations. Different methods have been used to collect the milt, through abdominal massage in anesthetized live fish (Akchay et al., 2004) or hormonally stimulated to spermiation (Glogowski et al., 1999) to others where the sperm is obtained straight from previously killed fish. In some species like black grouper (*Epinephelus malabaricus*), sperm should be collected in old animals (Gwo, 1993) with a specific technique of intra-testicular

maturation, to avoid contamination by urine and aging of sperm.

There are four main differences between mammalian and fish sperm.

1. the lack of acrosome in fish sperm, 2. a minimal mitochondria, allowing a very short period of motility, 3. motility is easily triggered (by osmotic change) and 4. lipid phase transition of fish sperm membranes, occur at lower temperatures making them less sensitive to cold shock (Zhang, 2004).

Since the first work of Blaxter in 1953 more than 200 fish species have been studied (Rana, 1995). Sperm from many fish species have been successfully cryopreserved with different levels of success. Most of the species studied are those with commercial value such as the *salmonidae* (Scott and Baynes, 1980) the *cyprinidae* (Horvath and Urbanyi, 2001) and *siluridae* (Tiersch, 2000) families, other research models like zebrafish (*Danio rerio*) and medaka (*Oryzias Latipes*) were also studied. Generally, marine species sperm has been more successfully cryopreserved than that of fresh water fish.

The effect of the freezing/thawing process on sperm needs to be assessed in terms of post-thaw sperm motility, percentage of live spermatozoa, cell viability and fertilization capacity (hatching rates). Many researchers have confirmed sperm damages are associated with loss of motility, fertility rates, ATP content, mitochondrial state, and function (Cabrita et al., 1998).

The protocols devised are numerous and vary in temperatures, freezing rates, thawing rates, and the CPT used. The cryoprotectants more commonly used are DMSO, methanol, glucose, PG (Propylene Glycol), EG (Ethylene Glycol), glycerol, ethanediol and sucrose and dimethylacetamide in concentrations ranging from 5 to 20 %. As zebrafish has become a recognized fish model with thousands of congenital, transgenic, and mutant lines created. Thus an effective sperm cryopreservation protocol highly consistent and reliable is required for this purpose (Yang et al., 2007).

1.3.2 Oocytes

The path to the cryopreservation of the fish oocytes deals with several aspects such as the sensitivity to the cryoprotectant toxicity, the membranes permeability to water and

cryoprotectants, sensitivity to chilling and the conditions used for low temperature storage and thawing.

Oocyte development has been divided in five stages in the zebrafish *Brachydanio rerio,* based on morphological criteria and on physiological and biochemical events (Isayeva et al., 2004a).

Stage I (perinucleolus stage) In this stage the oocytes begin to grow and the first meotic division takes place as the oocytes move towards the prophase. Oocytes are located on the periphery of the ovarian lamellae forming cysts; don't exceed 140 μm in diameter.

Stage II (cortical-alveolar stage) The major event at this stage is the appearance of cortical alveoli on the periphery of the oocyte; the diameter is 140-340 of μm. The formation of a tipartite vitelline envelope is also prominent in this stage, since this part continues developing new layers until it builds thicker more complex external layers, called the zona radiate externa, the zona radiate interna and zona radiate interna 2.

Stage III (vitellogenesis) The diameter of vitellogenic oocytes ranges from 340 to 690 μm. Vitellogenesis is the longest phase of oocyte development and requires a great amount of nutrient input. During this stage the follicles become more opaque and the germinal vesicles completely obscure (Selman et al., 1993).

Stage IV (maturation) At this stage the process of 'meiosis is reinitiated, the germinal vesicle migrates toward the oocyte periphery... the nuclear envelope breaks down, the first meiotic division occurs, and the chromosomes proceed to second meiotic metaphase where they arrest; at this point, the oocyte becomes an egg' (Selman et al, 1993).

Stage V (mature egg) Mature egg is ready to be spawned into the fresh water and fertilised. In appearance is homogeneous, finely granular, and weakly basophilic and their diameter is 700-750 μm (Selman et al., 1993).

Recent studies on chilling sensitivity of zebrafish (*Danio rerio*) oocytes showed that zebrafish oocyte are very sensitive to chilling and their survival decreased with decreasing temperature and increasing exposure time periods. These results showed

that chilling sensitivity may also be one of the limiting factors for development of protocol of their cryopreservation (Isayeva et al., 2004a).

Studies carried out on cryoprotectant toxicity to zebrafish (*Danio rerio*) oocytes showed that toxic effect of cryoprotectant on oocytes generally increased with increasing concentration and methanol was the least toxic cryoprotectant (Plachinta et al., 2004). Studies on membrane permeability of zebrafish (*Danio rerio*) oocytes showed that the water permeability and cryoprotectant permeability obtained for stage III zebrafish oocytes are generally lower than those obtained with mammalian oocytes and higher than those obtained with fish embryos (Isayeva et al., 2004b).

Systematic studies in zebrafish oocytes cryopreservation using controlled slow cooling have been undertaken recently, some of them using new protocols to improve controlled slow cooling (Guan et al., 2006a). New viability assessment methods for zebrafish oocytes in stage III have been developed using Fluorescence Diacetate (FDA) staining, Propidium Iodide (PI) staining (Zampolla et al., 2006). Although some methods have been used to assessing fish oocytes viability as trypan blue (TB) staining and thyazolyn blue (MTT) staining, the limitations relating to their low sensitivity and their applicability for specific development stages. In this study, FDA+PI staining was tested on fish oocytes for the first time. The results showed the method is promising and may offer a new reliable way of assessing the viability of fish oocytes (Zampolla et al., 2006). The current mechanical method used for isolating oocytes is laborious and time consuming so develop an enzymatic separation method for zebrafish oocytes was carried out for stages I, II and III respectively (Guan et al., 2006b).

1.3.3 EMBRYOS

Cryopreservation of fish embryos and eggs has been consistently attempted for the last twenty years with many species such as rainbow trout (*Salmo Mykiss*) (Haga, 1982), brown trout (*Salmo Trutta*) (Erdahl and Graham, 1980), common carp (*Cyprinus Carpio*) (Zhang et al., 1989) and zebrafish (*Danio rerio*) (Harvey, 1983; Zhang et al., 1993; Zhang and Rawson, 1996).

Cryopreservation of embryos is significantly more complex than spermatozoa and they presents problems related to permeation and uniform distribution of cryoprotectants. Low membrane permeability of fish embryos prevents cryoprotectants from entering the yolk to prevent cryodamage. Unfortunately, the technologies developed for mammalian embryo cryopreservation so far, have not been successful for multi-compartment, highly differentiated and yolk-filled embryos, such as those of fish (Bart, 2000). Survival of eggs and embryos after cryopreservation has not been achieved yet, as they have only survived for a short period at subzero temperatures so far.

1.3.4 BLASTOMERES

In fish species, cryopreservation of embryonic cells (blastomeres) can be an alternative to embryo as survival was established in several species (Harvey, 1983; Leveroni and Maisse, 1998, 1999; Strussmann et al, 1999; Blesbois and Labbe, 2003). Embryonic cells can be used through recipient embryos to produce chimeric embryos and such chimeric embryos can be used to produce future progeny of the donor genotype (Hong et al., 1998) to maintain the maternal genome diversity (Nakagawa et al., 2002).

The survival rate of fish blastomeres depends on the stage of blastomere, cryoprotectant used, and the cooling and thawing conditions. Where the higher survival rates were obtained from rainbow trout at 6C stage (72 hours after fertilization), using DMSO and Propane 1, 2 diol and a slow cooling rate (Leveroni and Maisse, 1998, 1999), other successful reports came from common carp (*Cyprinus carpio*), medaka (*Oryzias latipes*), pejerrey (*Odonthestes bonariensis*) and whiting (*Sillario japonica*) (Harvey, 1983; Leveroni and Maisse, 1998, 1999; Strussmann et al., 1999). There are no reported studies in the literature on isolated zebrafish blastomeres.

The use of isolated zebrafish is a valid alternative to whole embryo cryopreservation (Leveroni and Maisse, 1998). The blastomeres are much smaller in size and relatively easy to obtain from most animal embryos; they have nuclear genome and maternal mitochondrial DNA, and they possess pluripotent capabilities in cell culture. The

embryonic cell isolates can be used to create clones that express the donor-derived genotype (Leopold, 2006). It has been demonstrated by Harvey (1983) that the cryopreservation of individual fish blastomeres from zebrafish embryos could survive freezing and thawing at 50% epiboly or above.

Stage-dependent chilling sensitivity has been reported for many species of fish embryos. Most of these studies reveal that developmental stages beyond 50% epiboly are less sensitive to chilling (Zhang, et al., 2003). The zebrafish (*Brachydanio rerio*) was used as a model for basic studies of the chilling sensitivity, permeability and toxicity of cryoprotectants. In both intact and dechorionated embryos, early-stage embryos (1.25, 1.5, 1.75, and 2 h) were more susceptible to chilling injury at 0°C than late-stage embryos (50, 75, and 100% epiboly and three-somite stage) (Hagedorn, et al., 1997b). Zebrafish embryos were also shown to be less sensitive to late stage cryoprotectant toxicity (Zhang et al., 1993).

Other studies on the effect of external medium composition on membrane permeability of both intact and dechorionated zebrafish (*Danio rerio*) embryos in ultrasound studies were carried out. Earlier stage embryos were found to be more sensitive to ultrasound treatment and significant differences were found when embryos were treated for different time periods. The results showed that external medium composition has no effect on membrane permeability at early developmental stages. However, at later stages of development, embryos spawned into 30% HBSS were less permeable than embryos in system water, irrespective of calcium concentration. A decrease in permeability was observed with dechorionated embryos in 30% HBSS, however the effect was not significant (Zhang et al., 2005).
Recent research on fish embryo cryopreservation is focused on several new approaches: 1 permeabilisation of embryo membranes, through media modification and ultra-sound treatment, 2 the use of impedance spectroscopy for rapid assessment of embryo membrane permeability, 3 altering fish embryos with aquaporin-3 to improve permeability of the membranes to water and CPA's and, 4 direct modification of the yolk mass by micro-manipulation (Zhang et al., 2005).

Fish embryos are also prone to chilling injury unrelated to ice crystal damage. Their high chilling sensitivity, especially those in a developmental stage within the 50% epiboly, have been accounted for many fish species (Zhang, 2004). Chilling injury is

linked to the large quantity of lipids inside the embryo, as demonstrated in many mammalian species (Nagashima et al., 1994).

1.4 USE OF ZEBRAFISH (*DANIO RERIO*) AS A MODEL

The zebrafish was first used as a model by Dr. Georges Streisinger, a scientist working in developmental genetics at the University of Oregon, Washington, USA. Being a hobbyist he realized that zebrafish were small enough and with a short generation interval to allow keeping large numbers required for genetic studies. Transparent embryos are large enough to work with *in vitro* manipulations (Westerfield, 1995).

Zebrafish *Danio rerio* belong to the *Cyprynidae* family, they are tropical fish originally found in streams and pond near the Ganges River in India and Burma. Zebrafish have been extensively used as a model for studies in human genetics, disease and health.

ZEBRAFISH DEVELOPMENT

Zebrafish development is shown in Fig 1.2

When the **zygote period** starts (1-2 cells / 10-45 min), cytoplasm moves to the animal pole to form the blastodisc.

During the **cleavage period** (from 4 –64 cells / 1-2 hours) 6 cleavages occur. The cells, or blastomeres, divide synchronously on 15 minute intervals.

Blastula period (128 cells-50% epiboly / 2h15'- 5 h 15') at the 10th cleavage, the midblastula transition occurs. Transition cell divisions turn asynchronous after midblastula, embryo is formed by 3 cell layers: the enveloping layer (EVL), deep cells, and the yolk syncytial layer (YSL) formed from the fusion of cells adjacent to the yolk cells, the margin reaches 30% epiboly.

Gastrula period (50% - 100 % epiboly / 5h 20'-10 h) epiboly is driven by the migration of nuclei and cytoplasm in the YSL and attachments between the YSL and the EVL, movements of involution, convergence, and extension form the epiblast (that later will give rise to the ectoderm), hypoblast (that will form a mixture of endoderm and mesoderm), and the embryonic axis.

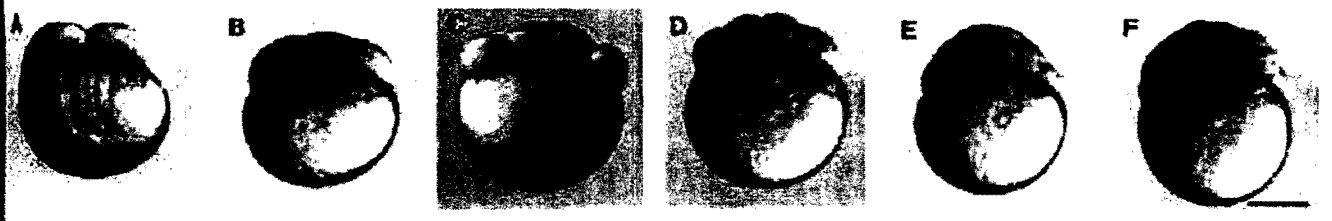
During the **Segmentation period** (3 – 26 somite / 10-24 h) somites, and neuromeres develop; early organogenesis and movements; the tail appears.

Pharyngula period (Prim 6 – High pec / 24-48 h) this is the stage where body axis straightens from its early curvature about the yolk sac, pigmentation and fin development start.

The last stage or **Hatching period** (Long pec – protruding mouth / 48-72 h)

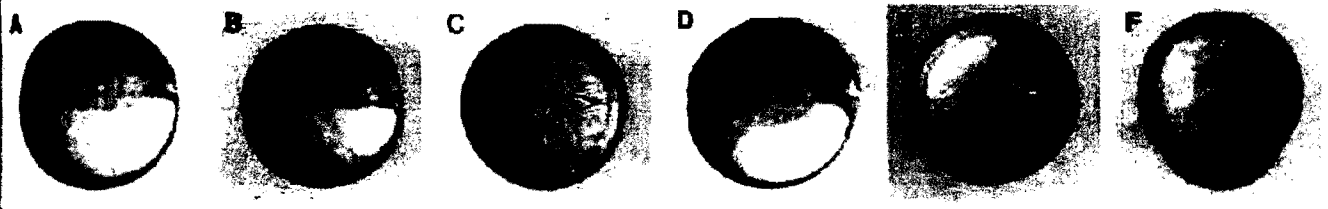
Means the complete morphogenesis of primary organs; cartilage development in head and pectoral fin; hatching occurs asynchronously. At 72 h, the swim bladder inflates the food-seeking and avoidance behaviours activa.

FIG 1.2 STAGES OF EMBRYONIC DEVELOPMENTS OF ZEBRAFISH (ZYGOTE, CLEAVAGE, BLASTULA AND GASTRULA PERIOD).

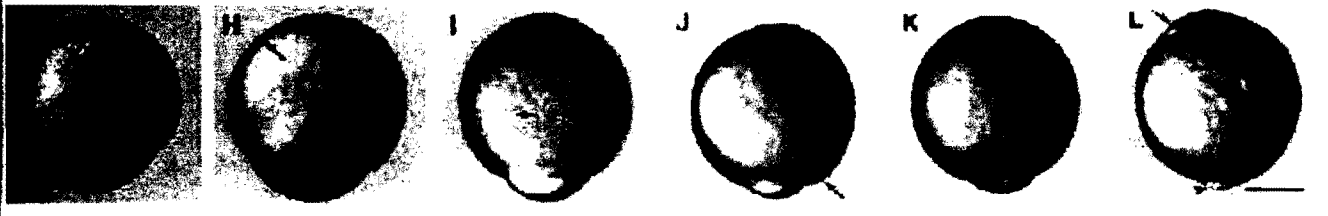


A: 2-cell stage (0.75 h). B: 4-cell stage (1 h). C. 8-cell stage (1.25h). D: 16-cell stage (1.5 h). E: 32-cell stage (1.75 h). F. 64-cell stage (2 h).

A: 256-cell stage (2.5 h). B: high stage (3.3 h). C. oblong stage (3.5 h). D. sphere stage (3.8 h). E: dome stage (4.3 h). F. 30%-epiboly stage (4.7 h).



A:50%-epiboly stage (5.25 h). B. Germ ring stage (5.7 h). C. Animal pole view germ ring stage D: Shield stage (6 h). E: Animal pole view shield stage F: 70%-epiboly stage (7.7 h).



G: 70%-epiboly stage H: 75%-epiboly stage (8 h). I: 80%-epiboly stage (8.4 h). J: 90%-epiboly stage (9 h). K: 90%-epiboly stage L: Bud stage (10 h)

(Scale bar: 250 μm. (Kimmel et al., 1995).

1.5 AIMS OF THE PRESENT STUDY

The aim of the project is to develop an optimum cryopreservation protocol for zebrafish (*Danio rerio*) blastomere. The protocol to be used is the optimum protocol that has been proved in previous experiments on zebrafish blastomere at 50% epiboly (Lin, et al., 2007).

Studies on toxicity of cryoprotectants will be carried out before freezing protocol development. Zebrafish blastomeres at 75% epiboly stages will be used, as they have not been studied previously.

Toxicity assessment will be carried out on several of the standard cryoprotectants such as: DMSO (dimethyl sulphoxide), methanol, E.G. (ethylene glycol) and P.G (propylene glycol), as well as the proposed novel CPA's: coffee, $NaHCO_3$ (sodium bicarbonate) and honey.

CHAPTER 2

MATERIALS AND METHODS

2.1 GENERAL METHODS

All the experiments were carried out at LIRANS Research facilities, University of Bedfordshire.

2.1.1 MAINTENANCE OF ZEBRAFISH (*DANIO RERIO*)

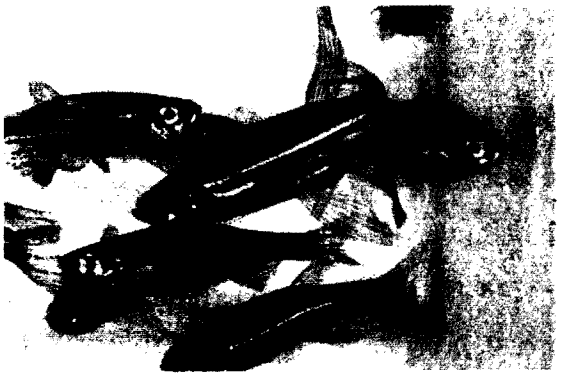


Fig 2.1 Adult males and females zebrafish

Adult zebrafish (2-3 months old) (Fig 2.1) were provided to LIRANS by Aquascope, LTD (Birmingham, UK), They were maintained in a 30 litres tank (28 x 28 x 58 cm) at 28°C, with tap water which was aged for 12- hrs before use. There are temperature control and filtration systems in each tank. Males and females were kept together with approximately 1 male for every 2 females (25 to 30 fish in each tank). The fish tank was cleaned weekly and over 80% of the water changed weekly with complete renewal every 4 weeks. Automatic light cycle control was set for light/dark = 12/12h. The impact of light-dark cycle known as circadian rhythms is important in determining the sleeping and feeding patterns of all animals. There are clear patterns

of brain wave activity, hormone production, cell regeneration and other biological activities linked to this daily cycle. An endogenous circadian clock which is constantly reset "entrained" by environmental factors such as light to ensure that it remains synchronised with the natural 24 hour cycle. Typically, the circadian clock in vertebrates was shown to be located in so-called central "oscillator" or "pacemaker" structures. The rhythm period can be reset by exposure to a light or dark pulse (Wikipedia). Some studies in fish shown that in the dark, the lymphocyte count decreased while the monocyte and basophil counts increased in the carp blood (Suquet et al., 2000).

2.1.2 FEEDING

Adult fish were fed three times a day with TetraMin flakes fish food (ingredients: fish and fish derivatives, cereals, yeast, vegetable protein extracts, mollusks and crustaceans, oils and fats, algae, various sugar, EEC permitted colorants and preservative) produced by Tetra, Germany. To enhance their breeding process the fish were also fed once a day with live adult brine shrimps (approx: protein 54%, oil 11%, ash 8%) purchased from ZM Ltd (Winchester). Excess of food in the water was removed. A holiday tablet fish food (ingredients: casein, fish meal, mussel meal, alder bark meal, colorant -iron oxide) produced by Sera-Germany, were put inside the tank at weekends and holiday periods.

2.1.3 BREEDING AND EMBRYO-COLLECTION

When female fish reach three months of age and the average length and weight of 24.9 mm and 1100 mg respectively, they are ready to start spawning (Piron, 1978). Each female fish spawns between 200 and 600 eggs per event, in some very well fed zebrafish raised with optimal conditions, spawns of 800-1200 have occurred. Zebrafish breed daily shortly after dawn, or after light is turned on in captivity. Embryos were collected from a glass tray covered by a plastic net. Embryos were then kept in beakers at 28°C water tank until they reach the 75 % epiboly stage (~8hrs after fertilisation).

2.1.4 DECHORIONATION OF THE ZEBRAFISH EMBRYO AND ISOLATION OF BLASTOMERE

This experiment was carried out using 75% epiboly stage zebrafish embryo because studies demonstrated that later stages embryos were less chilling sensitive than 50% epiboly stage or earlier stages (Zhang and Rawson, 1995). Studies carried out on other aquatic species-marine (whiting, *Sillago japonica*); estuarine (pejerrey, *Odontesthes bonariensis*); and freshwater (medaka, *Oryzias latipes*) have also shown that later stage blastomeres are less chilling-sensitive. The highest rates of successful cryopreservation were observed with older rather than younger blastomeres (Strüssmann et al., 1999). 75% epiboly stage zebrafish blastomeres have not been studied before and therefore were used in the present study.

Fig 2.2 75% epiboly stage zebrafish embryo

Once the embryos reached 75 % epiboly stage (Fig 2.2), dead embryos were removed. Viable embryos were placed in a petri dish and the tank water was replaced with PBS (1 phosphate buffered saline tablet was dissolved in 200 ml deionised water). Embryos were dechorionated with sharp forceps. Embryos were repeatedly pipetted to separate the blastoderm and the yolk. Mixture of blastoderm and yolk (3ml) were then washed 3 times with PBS at 3000 rpm for 5 min. using a centrifuge (Eppendorf - Netheler-Hinz GmbH). Number of embryos used was from 500 to 1000 for each

experiment with 3 replicas. The experiment was repeated at least 3 times. Blastomeres washed with PBS were kept at room temperature as controls.

2.1.5 CHEMICALS

2.1.5.1 COMMONLY USED CRYOPROTECTANTS

Four cryoprotectant agents were used in this project: DMSO (dimethyl sulphoxide), PG (propylene glycol), EG (ethylene glycol) and methanol. The sources and purity of these cryoprotectants are listed in Table 2.1

This four cryoprotectants are commonly used in cryopreservation studies. Plachinta, et al., (2004) studied the toxicity of DMSO, methanol, ethylene glycol (EG), propylene glycol (PG), sucrose and glucose in zebrafish (*Danio rerio*) oocytes. Cryoprotectant methanol and glucose were used in cryopreservation of zebrafish (*Danio rerio*) oocytes by Guan, et al., (2008). Additionally, initial studies on cryopreservation and gene expression in isolated blastomeres of zebrafish (*Danio rerio*) using DMSO, methanol, PG and EG as cryoprotectants also were studied (Lin, et al., 2007).

Compound	Source	Purity
DMSO	SIGMA – ALDRICH	99.9% minimum
Propylene Glycol (PG)	BDH Limited Poole, England	99.5% minimum
Ethylene Glycol (EG)	BDH Limited Poole, England	99.5% minimum
Methanol	SIGMA Chemical Co.	99% minimum

Table 2.1 Sources and purity of the cryoprotectant used in this project

2.1.5.2 OTHER CRYOPROTECTIVE CHEMICALS AND COMPOUNDS

Some natural products are widely used in Herbal Medicine because of their therapeutic properties. Herbal Medicine has long been used for improving human helath. In order to find out wether these natural products have any cryoprotective effect on cells, some well-known natural products such as coffee and honey were used in the present study as numerous studies have demonstrated their effectiveness in protecting cells under stress.

Concentrations of the natural products were prepared according to minimum measure suggested in Natural Medicine with the purpose to amplify its effect; this can be prepared as an *extract* which is made by soaking the botanical or natural product in a liquid that removes specific types of chemicals.

Three products: Coffee (Arabica and Robusta beans, medium roasted, Sainsbury, UK), $NaHCO_3$ (SIGMA Chemical Co.) and honey with Blue-Green algae spirulina (Akasha Products Mexico) were used in this study.

The mechanisms of action of CPAs in cells are not fully understood, however they share properties that can prevent cellular injury during cryopreservation. The aim of introducing natural products in this study is to investigate whether theses products have cryoprotective properties.

2.1.5.2.1 HONEY

Honey has been around for thousands of years. It was used by ancient Egyptian as a sweetener, and in the mummifying process. Richard Evershead and Stephen Buckley of the University of Bristol, found that embalmers used a wider variety of ingredients on mummies from the Pharaonic (or dynastic) period. They used a combination of gas chromatography and mass spectrometry techniques because their ability to analyse small samples. The researchers found that embalming fluids contained resins from conifers and beeswax — the amounts of which increased in later mummies, possibly because of their antibacterial properties. Salts, resins, cedar oil, palm wine, myrrh,

cassia, gum, honey and bitumen all worked by drying the body out or fighting microbes (http://www.abc.net.au/science/news/stories/s399494.htm).

The Greeks discovered honey's healing potential and used it as medicine. Honey was routinely prescribed by physicians for the treatment of the respiratory system, and throat irritations. Honey was widely used as a natural antibiotic and to dress wounds as a base for healing unguents. Recent research has highlighted the antibiotic properties of manuka honey, as it offers an alternative therapy to the usually resistan methicillin- resistant Staphylococcus aureus, according to the American Society for Microbiology Toronto. In this case, the manuka honey prevents the damaging cell from completing its cycle showing "astonishing" antibacterial properties (Vastag, 2007, Knox, 2004).

The specific composition of any batch of honey will depend largely on the mix of flowers available to the bees that produced the honey. Honey is an excellent source of potassium, it also contains thiamin, riboflavin, niacin, pyridoxine and ascorbic acid, along with calcium, copper, iron, magnesium, manganese, phosphorous and sodium.

Other researchers have identified the flavonoids in honey, particularly caffeic acid and ferulic acid, as the most likely contributors. Carbohydrates, proteins and amino acids, vitamins, minerals and antioxidants and other compounds are just some of the 174 compounds (shown in table 2.2) have been identify for honey.

Studies on honey have demonstrated how this versatile compound can have excellent "antibacterial, antifungal, and wound-healing" promotion properties among other valuable properties (Osman et al., 2003. The study of wound biopsies has shown a correlation between the thicker epithelium surface with an increment in fibrolastic reaction and a higher deposition of collagen fibres. Experimental study therefore shows that the healing process of wounds is faster and smoother with honey in comparison with "silver-suphadiazine-treated wounds". A gastric cytoprotective property of natural honey in humans has also been reported as healing quality of honey (Kamel et al., 2002).

Although honey has been available as a natural remedy throughout history, it is not until recently that its healing properties are being made available at hospitals to help the management of infected wounds. Honey is now used by doctors in some hospitals

to treat the skin wounds patients receiving radiation therapy or as an antibiotic where pharmaceutical products do not respond. As a result it may seem as a reasonable option to consider using honey at device exit sites (Molan et al., 2004).

The enzyme glucose oxidase helps create hydrogen peroxide from glucose in honey (NHB, 2008), most of the antibacterial activity of the honeys occurs due to hydrogen peroxide generation The concentration of chemicals found in the honey applied to wounds is about 1000 times lower than the traditionally applied pharmaceuticals. This, as a result, prevents tissue damage while the hydrogen peroxide is constantly replaced by its catalysation from glucose (Molan, 1999).

Research has shown that honey can also stop the growth of bacteria (like MRSA) even when diluted seven to 14 times, when its sugar content is no longer effective. Some of the healing powers of honey have been attributed to the presence of "protein-munching enzymes", such as Hydrogen peroxide, which is known to act on dead or dying tissue by dissolving it and freeing space for healthy tissue to grow. It stimulates the growth of blood vessels, increasing oxygen and nutrients in the area, and augments the number of fibroblasts, which improve the tissue connectivity (Molan, 1999).

The work of French et al. (2005) concludes "typical honeys are about eight times more potent against coagulase-negative staphylococci than if bacterial inhibition were due to their osmolarity alone". This result supports significantly the role of honey as an active and powerful bacteria inhibitor, among other healing properties.

The chemical composition of honey affects several of its physical characteristics. Honey has a high refractive index of about 1.49 and a high viscosity, of approximately 120 poise at room temperature for clover honey, compared to a lower 0.0089 poise for water. (Ball, 2007). "The specific gravity of honey is about 1.4, and the specific heat of honey is about 40% less than that of pure water" (Graham 1992). Most of the carbohydrates in honey are monosaccharides, with more fructose than glucose. At a distant third place is sucrose; other disaccharides present in honey, albeit in very small quantities, are maltose, isomaltose, nigerose, turanose, and maltulose. At about 1% or less of the total sugars, a small quantity of higher sugars, oligosaccharides, and dextrins are also present in honey. Honey contains a number of acids which include amino acids (0.05-0.1%) and organic acids (0.17-1.17%). The

average pH of honey is 3.9 (with a typical range of 3.4 to 6.1), which is equivalent to a 0.0001 M aqueous solution of a strong monoprotic acid (Williams 2003).

The composition of honey can vary depending on the different sources of nectar. The low moisture content of honey is one of its defining characteristics, particularly since a medium with such a high osmotic pressure is not suitable for bacteria to survive. This is also one of the reasons why honey is the only food that will not decompose in a natural environment (Baker et al., 1982).

The main enzymes contained in honey in small amounts are invertase (a-glucosidase), diastase (amylase) and glucose oxidase. Also present in smaller amounts are catalase and acid phosphatase (NHB, 2008).

Studies on chilling sensitive in zebrafish (*Brachydanio rerio*) showed the presence of sucrose or trehalose enhanced cooling tolerance of the embryos.

Table 2.2 Honey Composition

Component	**Average (%)**	**Range (%)**
Water	17.2 —	12.2–22.9
Fructose	38.4 —	30.9–44.3
Glucose	30.3 —	22.9–40.7
Sucrose	1.3 —	0.2–7.6
Other disaccharides	7.3 —	2.7–16.0
Higher sugars	1.4 —	0.1–3.8
Gluconic acid	0.57 —	0.17–1.17
Acids (not including gluconic)	0.43 —	0.13–0.92
Lactones	0.14 —	0.0–0.37
Minerals	0.17 —	0.02–1.03
Nitrogen	0.04 —	0.0–0.13

Mineral	Average (ppm)	Range (ppm)
Potassium	205	100–588
Sulfur	58 0	36–108
Chlorine	52	23–75
Calcium	49	23–68
Phosphorus	35	23–50
Magnesium	19	11–56
Sodium	18 0	6–35
Iron	2.4	1.2–4.8
Copper	0.3	0.14–0.70
Manganese	0.3	0.17–0.44

Vitamin	Concentration (ppm)
Riboflavin	0.63
Pantothenic acid	0.96
Niacin	3.2
Thiamin	0.06
Pyridoxin	3.2
Ascorbic acid	22

Water Content	Viscosity (poise) at 25°C
15.5 %	138.0
17.1 %	69.0
18.2 %	48.1
19.1 %	34.9
20.2 %	20.4

Temperature (°C)	Viscosity (poise) at 25°C
13.7	600.0
29.0	68.4
39.4	21.4
48.1	10.7
71.1	2.6

Floral Source	Viscosity (poise) at 25°C (16.5% H2O)
Sage	115.0
Clover	87.5
White Clover	94.0

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According to the size and complexity of their molecules, sugars can be classified as follows.

1. monosaccharides as Levulose and Dextrose which are simple sugars and they are the predominating sugars in honey. 2. disaccharides, the best-known are sucrose (table sugar) isomaltose and maltose. 3. higher sugars are more complex sugars made up of three sugars, four or more monosaccharide units such as melezitose, erlose, kestose, raffinose, and dextrantriose.

Honey contains 7.3% of other disaccharides such as sucroses which is a nonreducing crystalline disaccharide made up of glucose and fructose; isomaltose (named also dextrinose) is an isomeric form of maltose formed by the action of maltase on glucose found in natural substances, and maltases that is an enzyme that hydrolyzes a-glucosides to glucose (White 1961).

Moreover various sugars have been used as cryoprotectors: such as sucrose, glucose,

trehalose, maltose, melezitose and raffinose were tested as cryoprotectants (Toshiaki, et al., 2007; Takahashi and Kanagawa 1985; Duarte, et al 1999; Nsabimana, et al., 2006).

In this study 95% Pure Natural Blossom Honey and 5% blue-green algae (*Spirulina platensis)* were used. Akasha Products, Made in Mexico. The honey that was used for the experiment is a trademark product, which has been used in alternative medicine to treat skin problems.

2.1.5.2.2 COFFEE

Protective effect of caffeine against neurodegeneration in a model of Parkinson's disease in rat has been reported. "The results demonstrated that caffeine administration for 1 month could attenuate the rotational behaviour in lesioned rats and protect the neurons of SNC against 6-OHDA toxicity" (Joghataie et al., 2004).

Coffee is also known to be toxic at high concentrations. According to a study by Sardão et al. (2002), cardiovascular disease and reproductive disorders are among some of the observed clinical conditions of high concentration beverages like coffee and tea. The study also notes that more research is needed on the exact toxic effects of caffeine on heart mitochondria in order to fully understand the risks involved. In addition, the influence of caffeine of the mitochondrial permeability transition needs further clarification according to the study.

Coffee compounds consist of carbohydrates, nitrogenous components, chlorogenic acids, volatile components, and carboxylic acids (Flament, I. 2001).

Carbohydrates

The three major constituents of carbohydrates are polysaccharides, mono and disaccharides and phytate. Polysaccharides are water soluble and are derived from pectic, galactomannans and arabinogalactan substances Monosaccharides and disaccharides are low molecular weight sugars composed by arabinose, glucose, galactose and mannose, which have small amounts of sucrose, fructose and rhamnose.

The content of phytate carbohydrates varies as it is offered percolated or as instant coffee.

Nitrogenous Components

Coffee contains two main groups of compounds: alkaloids and trigonelline, both groups having amino acids, nicotinic acid and proteins.

Caffeine

Caffeine is the most studied and more controversial alkaloid found in coffee since its discovery in 1820. The caffeine content in coffee is 1-2% on a dry weight basis. In a study by Kopeika et al., (2003) it was reported that the addition of caffeine to loach embryos significally increased their survival after the cryopreservation of the sperm.

Trigonelline

Trigonelline is a naturall constituent of green coffee (approximately 1%), which is known to create nitrogenous material like pyridines and pyrroles when roasted. It has been reported that it has antitumour activity and that it is thermally unbalanced.

Chlorogenic Acids

Chlorogenic are a form of acids formed by trans-cinnamic and quinic acid, in its most common form as combination of quinic acid and caffeic acid. The amount of Chlorogenic acid available in coffee can vary from 70mg to 350mg for a 200ml cup of coffee, depending on whether it is arabica or robust (CSIC, 2008). Chlorogenic acid plays an important role as inhibitor of tumors and by preventing cardiovascular disease, diabetes and mellitus (Morton, 2000).

Volatile Components

The volatile material present in coffee can be analysed through Gas Chromatography, for example, by studying the vapour directly above the coffee being tested.

Carboxylic Acids

The non-volatile acid content in coffee is less than 2% and contains small amounts of citric acid, malic acid, tartaric acid and oxalic acid. The acids presence gives the

coffee infusions its unique flavour. Research shows that variations in the pH of the substance can lead to "ionisation of functional groups (Phenolic hydroxy groups) and this can alter the flavour of the product". The thresholds of acids present in coffee as an aquous solution are less than 10 ppm (Lancashire, 1995). It has been found more than 30 different types of aliphatic acids in roasted coffee. These aliphatic acids include non-volatile monocarboxylic acids as well as volatile ones. Generally, a lower acid content is found in the darker roast of coffee (Arnaud, 1985).

In this project the concentration used was 0.1% (w/v) 0.1g of coffee in 100 ml PBS.

2.1.5.2.3 SODIUM BICARBONATE

Sodium bicarbonate ($NaHCO_3$) is also known as baking soda, saleratus or bicarbonate of soda. It is soluble in water. The natural mineral form is known as nahcolite.

The reaction of acids with sodium bicarbonate is a common method for neutralizing acid spills. A wide variety of applications follow from its neutralization properties including ameliorating the effects of white phosphorus in incendiary bullets, from spreading inside a soldier's afflicted wounds. It is commonly used to increase the pH and total alkalinity of the water for pools and spas. Sodium bicarbonate solution can be used for restoring the pH balance of water that has a high level of chlorine. It is also sometimes used in septic tanks to control pH and bacteria.

The concentration used in this project was 0.0006 % (w/v) 0.06g $NaHCO_3$ in 100 ml of PBS.

CONCENTRATIONS OF CRYOPROTECTANTS/SUPPLEMENTS USED

The history of homeopathy goes back to the eighteenth century, where the German physician Samuel Hahnemann began studying the basic principles of homeopathy. Hahnemann established the rules necessary to produce homeopathic medicines, where

the extremely low doses used are more effective than larger doses. In the UK, homeopathic remedies have been used for more than 200 years, while in the world of science, they have laid the foundations for the production of pharmaceutics still used today (Homeopathy, 2008).

The preparation of homeopathic medicines is regulated by the European Pharmacopaeia in the European Union, and by other organizations worldwide. The active micro-doses found in homeopathic substances contain botanical and biological substances and minerals, including: NaCl (table salt), Quartz, *Apis mellifera* (honey bee) and *Calendula officinalis* (marigold) among others (MEB, 2008).

Cryoprotectants are generally used in high concentrations to achieve an effective cryoprotectant action but regrettably this has an adverse effect, as this high concentration can some times be toxic if not lethal for the cells (Pillai, et al 2001; Hee-Jun, et al., 2002). Considering this, it seemed to be worth it to assess the effect of lower concentrations of the natural products that were to be used as experimental CPA's.

From previous experience when preparing low doses in therapeutic work it was understandable that very low concentrations need to be used in homeopathy and herbal remedies where the toxicity of the natural product needs to be reduced to a minimum, as it is crucial to the success of the treatment.

In some studies as those done on the effect in cancerous human lymphocytes where toxicity has also been a fundamental issue, the application of extremely low concentrations has been attempted to keep variables with very short intervals (Walchli, et al., 2006).

Several studies have shown that dilution of honey increases significantly its antibacterial properties as the production of hydrogen peroxide (one of the main antibacterial agents) augments considerably on dilution (White et al., 1963). Recent studies have reported that highly diluted honey increases the antibacterial activity of honey by slowly releasing the antiseptics while not damaging the tissue involved (Olaitan, 2007; Al Somai, 1994). Other studies have revealed that the antibacterial activity of honey increases at much lower concentrations, for example, 0.25% (v/v)

(D'Agostino, 1961); 1.5% (v/v) (Dustmann, 1979); 0.6% (v/v) (Buchner, 1966) and 1.5% (v/v) (Christov and Mladenov, 1961) (Molan, 2001).

The glucose oxidase enzyme present in honey becomes more functional as honey is diluted, by releasing hydrogen peroxide at low and constant intervals; this process enhances considerably honey's antibacterial action (Cimolal, 2007). A study on seven different species of bacteria found that honey could be diluted 'nearly ten-fold' and still show important antibacterial action (Molan, 1996). Molan (1996) also found that Manuka honey could be diluted up to 54 times its original volume and still inhibit completely the growth of Staphylococcus *aureurs* bacteria.

Tonks et al. (2001) reported that honey concentration of 1% can 'activate the many facets of the immune response to infection', while a very low concentration of 0.1% can stimulate the development of lymphocytes in the cell culture and activate the role of phagocytes in the blood (Abuharfeil et al., 1999). These mechanisms are a positive function of the agents in honey because they can enhance the immunity of the organism involved (Ryan and Majno, 1977). After reflecting on these results it can be deduced that decreasing the concentration of honey by a significant amount can be highly beneficial for the tissues or organisms involved, due to its protective properties.

Highly diluted honey solution as a novel cryoprotectant was therefore used in the present study at concentrations of 0.043% (w/v) of honey.

2.1.5.3 OTHER CHEMICALS

Other chemicals used in the project are Trypan blue 0.4% (TB) solution (SIGMA Chemical Co), Ethanol absolute (SIGMA – ALDRICH) and Phosphate buffered saline (PBS) tablets (Fluka Biochemika).

2.2 STUDIES ON CRYOPROTECTANT TOXICITY TO ZEBRAFISH BLASTOMERES

Blastomeres (45µl in PBS) were added to 45 µl of double concentration of cryoprotectant solution at room temperature for 30 min. Blastomere toxicity was then assessed using trypan blue staining.

2.3 STUDIES ON CRYOPRESERVATION OF ZEBRAFISH BLASTOMERES USING CONTROLLED SLOW COOLING

45 *µl* of Blastomeres were incubated in (45 *µl* of 2M methanol in and 2M DMSO for 30 min at room temperature before were loaded in straws (90 *µl* in each). They were then placed inside a controlled rate freezer (Planner Kryo 550-16).

The following freezing protocol was used:

(1) starting temperature was set at 20.0°C, and subsequently cooled to -7.5°C at cooling rate of 2°C /min; (2) seeding at -7.5°C and samples were held at this temperature for 10 min, (3): samples were cooled at 1.0°C/min to the temperature of –40.0°C, (4): subsequently 5.0°C/min to -80.0°C holding the samples for 5 min before plung them into liquid nitrogen for at least 15 minutes. After freezing the blastomeres loaded in straws were quickly thawed in water bath (28°C) for 10 sec and were taken one by one for trypan blue (TB) viability assessment under a fluorescence microscope at room temperature. All the straws were assessed by TB staining after thawing and CPT removal followed by 3 different incubation periods (5 min, 30 min and 1 hour) and each sample was added double amount of PBS (90 *µl*, 180 *µl* and 360*µl*) for 5 min and this procedure was repeated 3 times. CPT was removed stepwise. The samples were then spun down 3 times at 3000 g /min for 5 min. Supernatant was then removed and fresh PBS added to samples and blastomere viability was assessed using TB staining. This cryopreservation protocol was used as it was demonstrated to be the optimal protocol for cryopreservation of 50% epiboly stage zebrafish blastomeres by LIRANS.

2.4 VIABILITY ASSESSMENT

Trypan blue staining was used to assess blastomere viability following 30 min exposure to cryoprotectants in toxicity tests and after cryopreservation Trypan blue solution (0.4%, Sigma) was added to equal volume of blastomeres suspension. Blastomere viability was evaluated using microscope in terms of the percentage survival.

Trypan blue is a vital stain that is used to colour dead tissues or cells blue. It is a diazo dye and its quick and easy to use. Live cells or tissues with intact cell membranes are not coloured. Since cells are very selective in the compounds that pass through the membrane, in a viable cell Trypan blue is not absorbed; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue colour under a microscope. Since live cells are excluded from staining, this staining method is also described as a Dye Exclusion Method.

Trypan blue is commonly used in microscopy (for cell counting) and in laboratory for assessment of tissue viability. However, the method cannot distinguish between necrotic and apoptotic cells.

2.5 STATISTICAL ANALYSIS

The results were analysed using variance (ANOVA statistics). The student's *t*-test was applied to determine differences in results between two contrasting groups with the assumption that the differences of the paired samples follow a normal distribution. Values of *P* less than 0.05% were considered statistically significant ($p < 0.5$). Means, standard errors, and threshold values for *t*-test and ANOVA were calculated using Excel Version 7.0a for Windows.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 INTRODUCTION

In the last two decades, several attempts to cryopreserved fish eggs and embryos have been carried out on about 20 species; controlled slow cooling was employed in most of these studies. Although eggs or embryos have been shown to survive for a short time after cooling to subzero temperatures, successful cryopreservation of fish eggs and embryos remains elusive. The aim to this project is to develop successful cryopreservation protocols for zebrafish blastomeres as they can be used in material genome preservation.

In order to develop successful controlled slow cooling protocol for fish blastomere, it is important to consider some factors as a embryo developmental stage; cryoprotectant type and concentration; treatment time and methods; cooling rates; thawing rates and methods; and removal of cryoprotectants. Zebrafish blastomeres at 75% epiboly stage were used in this study as embryos at this stage are less sensitive to chilling than earlier stages (Zhang and Rawson, 1998).

Cryoprotectants such as: Dimethyl Sulphoxide (DMSO), Propylene Glycol (PG), Ethylene Glycol (EG) and Methanol have been used in fish embryo and blastomere cryoprotectant toxicity studies. Methanol was found to be more effective than DMSO (Zhang et al., 1993) in zebrafish embryos whilst DMSO was reported to be more effective than methanol in zebrafish blastomeres (Lin et al., 2007). In this project, toxicity studies were carried out for all four cryoprotectants (DMSO, methanol PG and EG). Blastomeres were incubated in cryoprotectant solutions for 30 min at 22°C. Blastomeres viability was assessed using trypan blue staining. After the selection of cryoprotectant, blastomeres viability was assessed after controlled slow cooling. Additional compounds were also used in cryopreservation studies including: coffee

0.1% (w/v), $NaHCO_3$ 0.0006% (w/v) and honey 0.043% (w/v). Blastomere viability was assessed immediately after freezing and after up to 2 hr incubation at 22°C.

3.2 RESULTS

3.2.1 STUDIES ON CRYOPROTECTANT TOXICITY

3.2.1.1 DIMETHYL SULPHOXIDE

Toxicity of three concentrations of DMSO (1M, 2M and 3M) to blastomeres before freezing was assessed. Control blastomeres were incubated at room temperature for 30 min in PBS in all experiments. Fig 3.1 shows blastomere survivals in 3 concentrations of DMSO after 30 min at room temperature. The results showed that no significant differences were found between control (98.7%) and 1M (98.3%) or 2M (97.8%) DMSO, however, 3M DMSO (89%) significantly reduced blastomere viability ($p < 0.05$) when compared with controls (98.7%). The mean number of cells per 1.5 ml in each sample was 904 (range 764-1061).

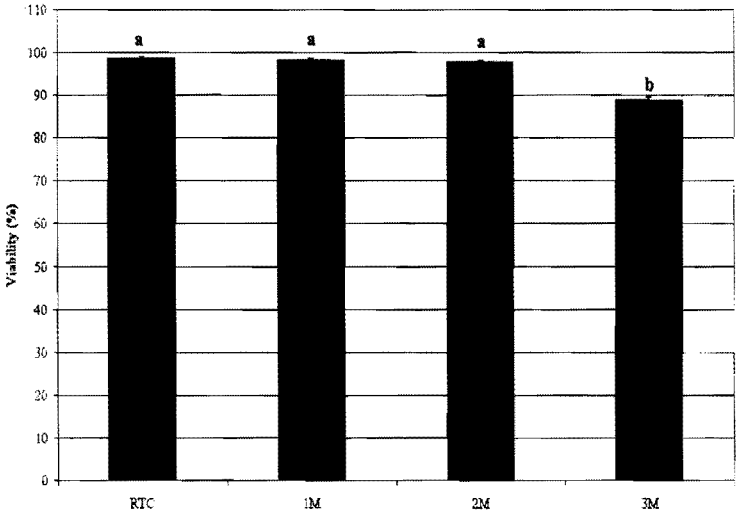

Fig 3.1 Survival of blastomeres at 75% epiboly stage after 30 min exposure to DMSO at room temperature. Different letters in the graph indicates significant differences. RTC = room temperature control. Error bars represent standard errors. Significant differences are indicated in different letters (n=3, $p>0.05$).

3.2.1.2 METHANOL

Methanol has been shown to be effective in zebrafish embryo cryopreservation therefore was used in this experiment. Blastomere viability was assessed using trypan blue staining. The results in Fig 3.2 showed that blastomeres survivals in 1M (97.1%), 2M (96.7%) and 3M methanol (92%) were significantly different from room temperature controls (98.4%) ($p>0.05$). The mean number of cells per 1.5 ml in each sample was 845 (range 715-948).

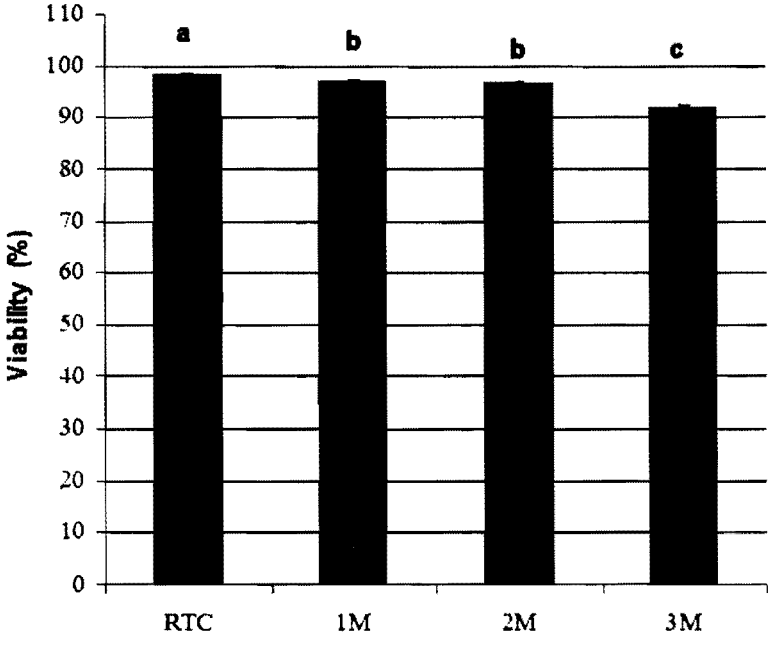

Fig 3.2 Survival of blastomeres at 75% epiboly stage after 30 min exposure to methanol at room temperature. Different letters in the graph indicates significant differences. Error bars represent standard errors. Significant differences are indicated in different letters ($n=3$, $p>0.05$). RTC = room temperature control.

3.2.1.3 PROPYLENE GLYCOL

In the Fig 3.3 Comparisons were made between blastomere survivals in different concentrations of propylene glycol, there are no significant difference between control (99%) and 1M treatment (98.9%), however in the 2M concentration the differences were significant ($p > 0.05$), (96.9%) blastomere survived when compared with room temperature control (99%). 3M significantly decreased blastomere survival to 91.7% ($p < 0.05$). The mean number of cells per 1.5 ml in each sample was 812 (range 752-860).

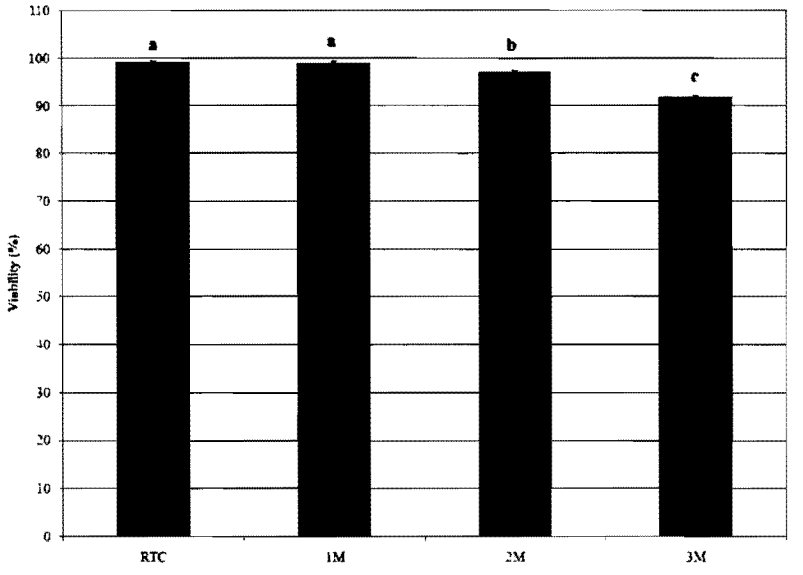

Fig 3.3 Survival of blastomeres at 75% epiboly stage after 30 min exposure to propylene glycol at room temperature. RTC = room temperature control. Error bars represent standard errors. Significant differences are indicated in different letters ($n=3$, $p>0.05$).

3.2.1.4 ETHYLENE GLYCOL

Studies with ethylene glycol (EG) in Fig 3.4 showed that no significant differences of blastomere survival were found between control (98.9%) and 1M (98.5%). However,

2M (97%) and 3M (93.2%) EG significantly reduced blastomere viability when compared with the controls ($p < 0.05$). The mean number of cells per 1.5 ml in each sample was 831 (range 814-844).

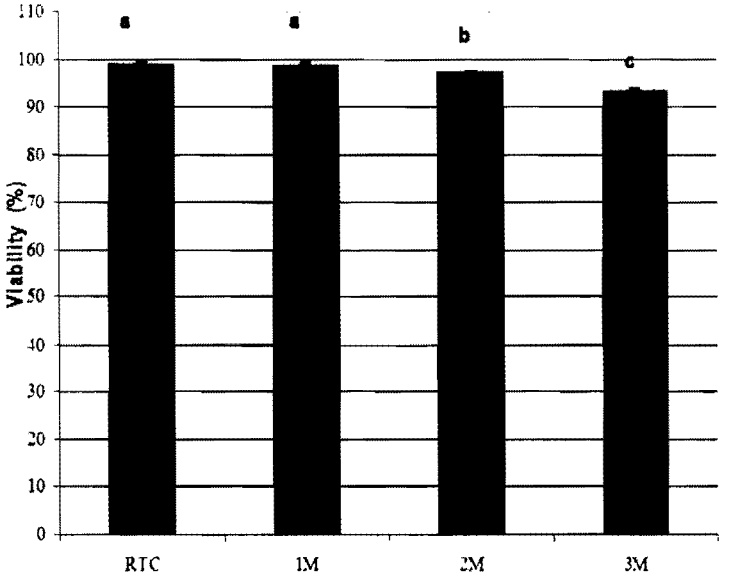

Fig 3.4 Survival of blastomeres at 75% epiboly stage after 30 min exposure to propylene glycol at room temperature. RTC = room temperature control. Error bars represent standard errors. Significant differences are indicated in different letters ($n=3$, $p>0.05$).

3.2.1.5 Comparison of toxicity of different cryoprotectants

Tables 3.1 Showed that the No Observed Effect Concentrations (NOEC) for methanol, DMSO, PG and EG were 2M, 2M, 1M and 1M respectively.

As 2M DMSO and 2M methanol were less toxic than propylene glycol and ethylene glycol, all the subsequent assessments were carried out using 2M DMSO and 2M methanol.

CRYOPROTECTANT	22°C
DMSO	2M
Methanol	2M
Propylene Glycol	1M
Ethylene Glycol	1M

Table 3.1 The No observed Effect Concentration (NOEC) of cryoprotectants to 75% epiboly blastomeres.

3.2.2 CRYOPRESERVATION OF ZEBRAFISH BLASTOMERES USING CONTROLLED SLOW COOLING

In this experiment, three compounds were investigated and their solutions were used as cryopreservation media: 0.043% (w/v) honey, 0.1% (w/v) coffee and 0.0006% (w/v) $NaHCO_3$. Blastomeres were also frozen using 2M DMSO and 2M methanol. Both room temperature control (RTC) and freezing control (FC, blastomere frozen in PBS) were used.

3.2.2.1 SURVIVAL OF BLASTOMERES IMMEDIATELY AFTER FREEZING (NO CRYOPROTECTANT REMOVAL)

Fresh solutions of $NaHCO_3$, coffee, and honey were prepared in: w/v % before experiments.

3.2.2.1.1 EFFECT OF NOVEL ADDITIVES AND $NaHCO_3$

The effects of cryoprotectants to survival of blastomeres from 75% epiboly embryos were variable (Fig 3.5). The results showed that no significant differences were found

between room temperature control 98.4% and 2M DMSO (98.2%), 2M methanol (97%), however $NaHCO_3$ 0.0006% (67.5%) significantly reduced blastomere viability when compared with controls (98.4%) ($p < 0.05$). The mean number of cells per 1.5 ml in each sample was 776 (range 688-905).

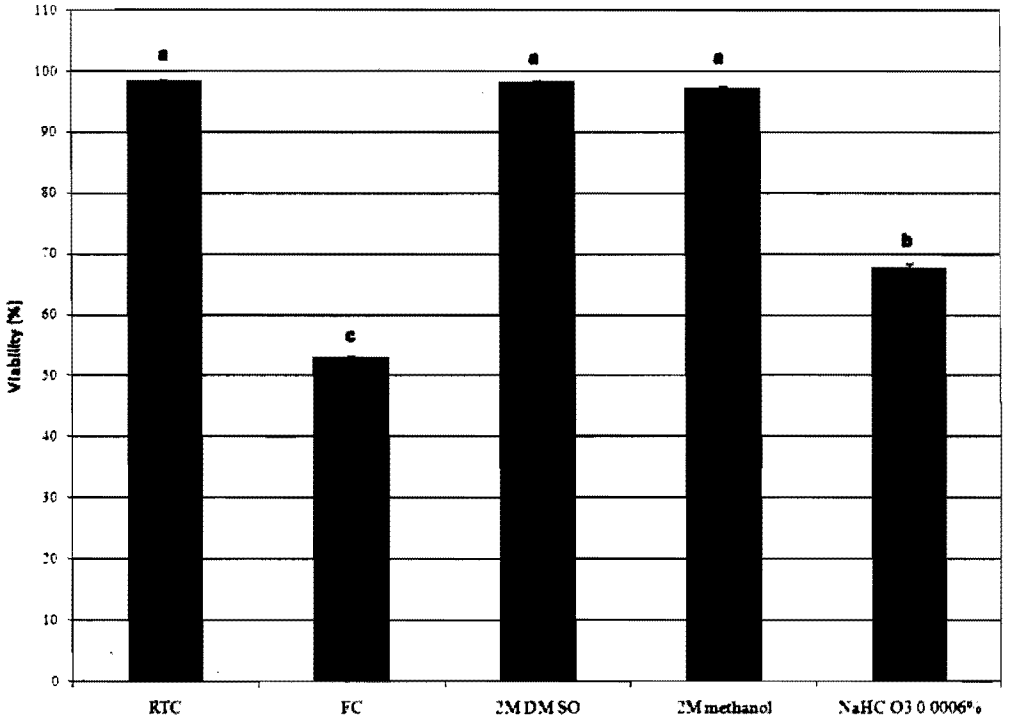

Fig 3.5 Blastomere survivals in DMSO, methanol and $NaHCO_3$ immediately after freezing with no cryoprotectant removal. Errors bars represent standard errors. Significant differences are indicated in different letters (n=3, $p>0.05$).

3.2.2.1.2 Effect of cryoprotectant and coffee

The results in Fig 3.6 showed that significant differences were found between room temperature control (98.9%) and 2M DMSO (96.6%), 2M methanol (92.6%) and 0.1w/v % coffee (52.7%). Survival of blastomeres in 0.1 % coffee decreased

significantly after freezing when compared with control and 2M DMSO or methanol ($p < 0.05$). The mean number of cells per 1.5 ml in each sample was 1167 (range 1043-1448).

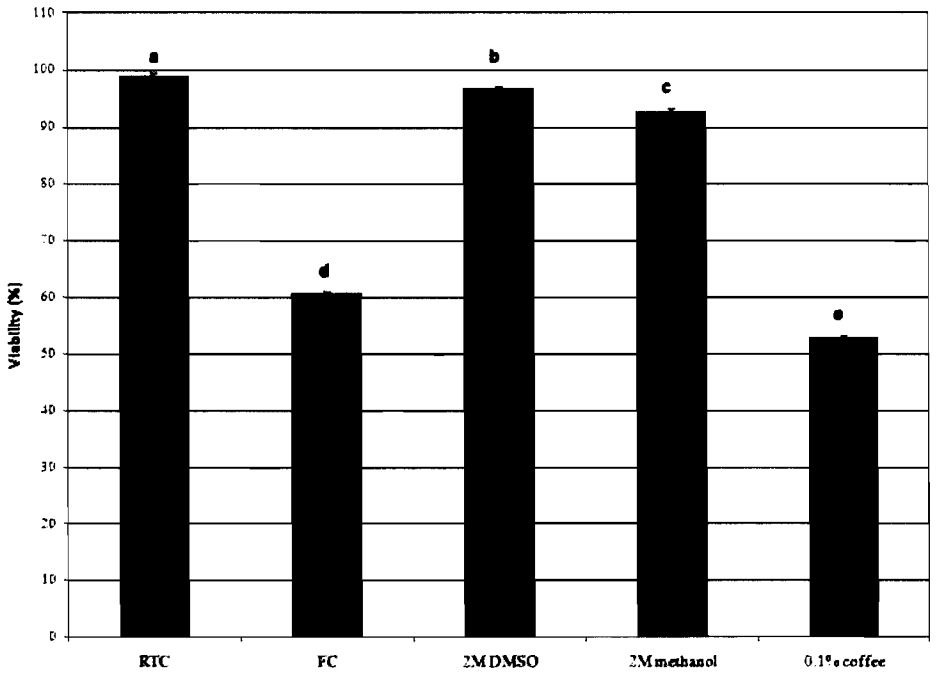

Fig 3.6 Blastomere survival in methanol, DMSO and coffee immediately after freezing with no cryoprotectant removal. Error bars represent standard errors. Significant differences are indicated in different letters (n=3, p>0.05).

Because significant differences were observed between blastomere survival in 2M DMSO (96.6%) and 2M methanol (92.6%), in the subsequent experiments 2M DMSO was used to compare with other compounds.

3.2.2.1.3 EFFECT OF CRYOPROTECTANT AND HONEY

Honey at 0.043 w/v % was used in these experiments. Fig 3.7 showed blastomeres viability immediately after freezing, the results of blastomere viability showed that no significant differences were found between room temperature control (98.6%) and honey (98%), although blastomere frozen in PBS (53.9%) and 2M DMSO (89.8%)

were significantly damaged when compared with controls at room temperature (97.3%) ($p > 0.05$).

The mean number of cells per 1.5 ml in each sample was 1092 (range 977-1246).

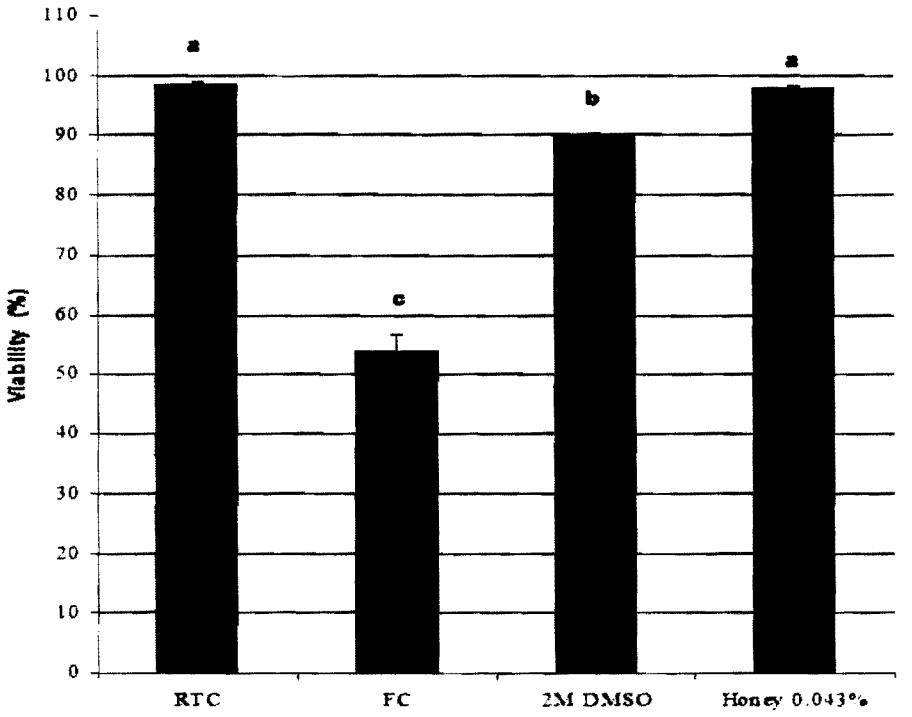

Fig 3.7 Blastomere survival in DMSO of earlier results and honey immediately after freezing with no cryoprotectant removal. Error bars represent standard errors. Significant differences are indicated in different letters (n=3, $p>0.05$).

COMPARISONS OF DIFFERENT NOVEL ADDITIVES AND CRYOPROTECTANT MEDIA

Results in Fig 3.8 showed there were significant differences between room temperature control (98.4%) and 2M DMSO (93.2%), 2M methanol (90.8%), 0.1% coffee (52.9%), and 0.0006% $NaHCO_3$ (72.9%) ($p > 0.05$), however 0.043% honey (98.1%) did not reduce blastomere viability when compared with room temperature control (98.4%) ($p > 0.05$). FC dropped (59.2%) immediately after freezing.

The mean number of cells per 1.5 ml in each sample was 909 (range 770-1125).

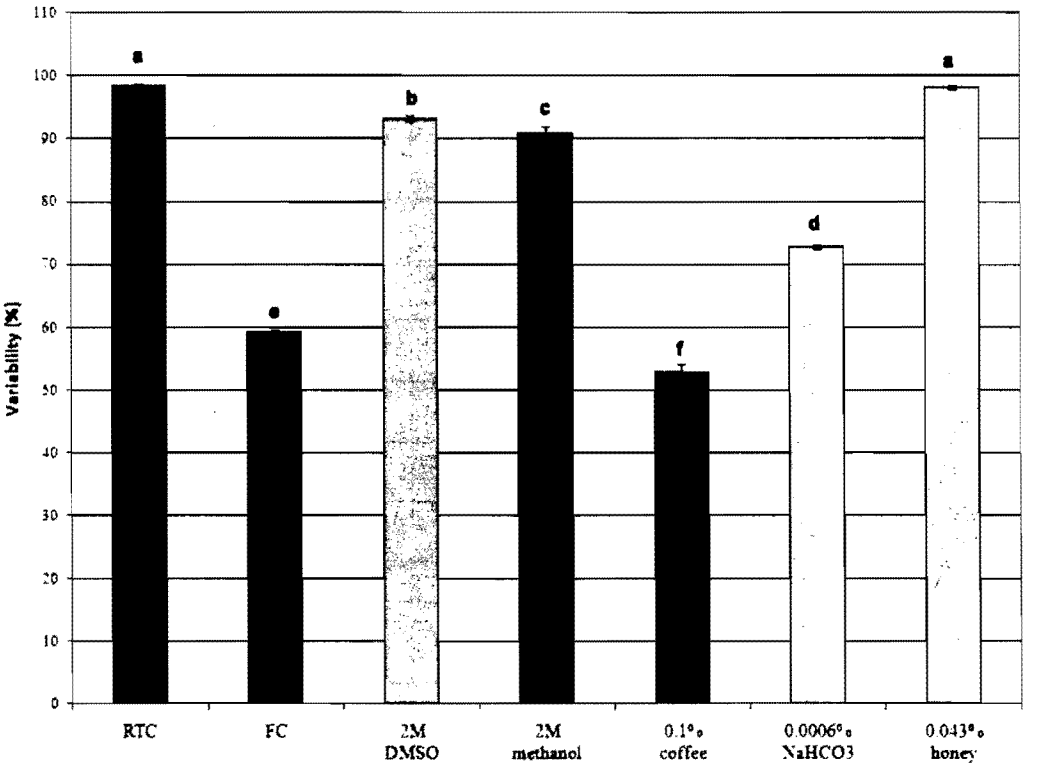

Fig 3.8 Comparisons of blastomere survivals in different media after freezing. Error bars represent standard errors. Significant differences are indicated in different letters (n=3, $p>0.05$).

3.2.2.2 Effect of extended incubate period on blastomeres survival after freeze-thawing and cryoprotectant removal

Comparisons of blastomere survivals after 30 min incubation at room temperature following freeze-thawing in 2M DMSO and 0.043% honey are shown in Fig 3.9. Blastomeres were washed three times in PBS after thawing. Blastomeres were then incubated in PBS at room temperature for 30 min.

Significant differences ($p > 0.05$) were found in blastomere survival between room temperature control (98.6%) and honey 0.043% (97.2%) and 2M DMSO (89.5%). The mean number of cells per 1.5 ml in each sample was 1118 (range 976-1364).

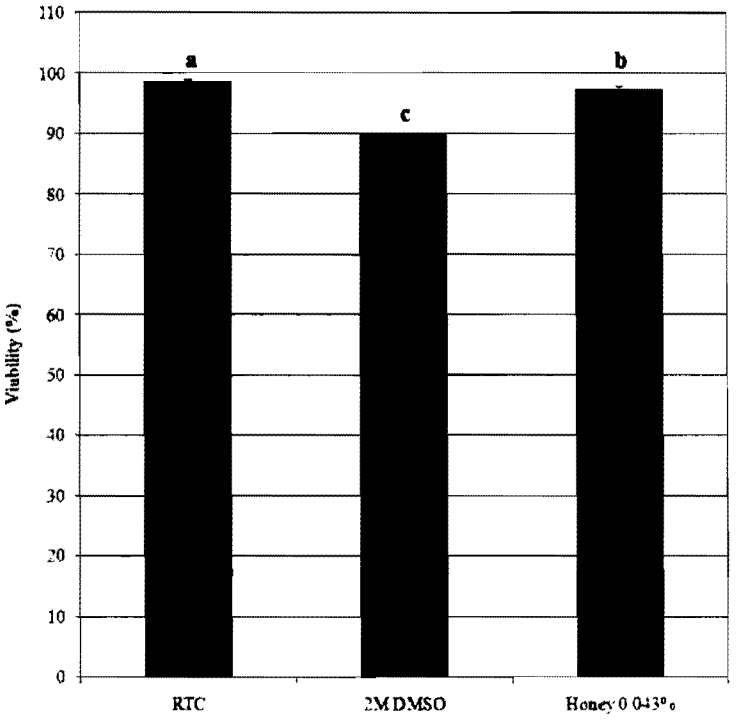

Fig 3.9 Blastomere survival after freeze-thawing and cryoprotectant removal. Blastomeres were incubated at room temperature for 30 min after cryoprotectant removal. Error bars represent standard errors. Significant differences are indicated in different letters ($n=3$, $p>0.05$).

Comparisons of blastomere survivals after 60 min incubation at room temperature following freeze-thawing in 2M DMSO and 0.043% honey are shown in Fig 3.10. Blastomeres were washed three times in PBS after thawing. Blastomeres were then incubated in PBS at room temperature for 60 min.

Significant differences ($p > 0.05$) were found in blastomere survival between room temperature control (98.6%) and honey 0.043% (97.2%). 2M DMSO (66.7%) also significantly decreased blastomere survival when compared with controls at room

temperature (98.6%) ($p > 0.05$). The mean number of cells per 1.5 ml in each sample was 1118 (range 976-1364).

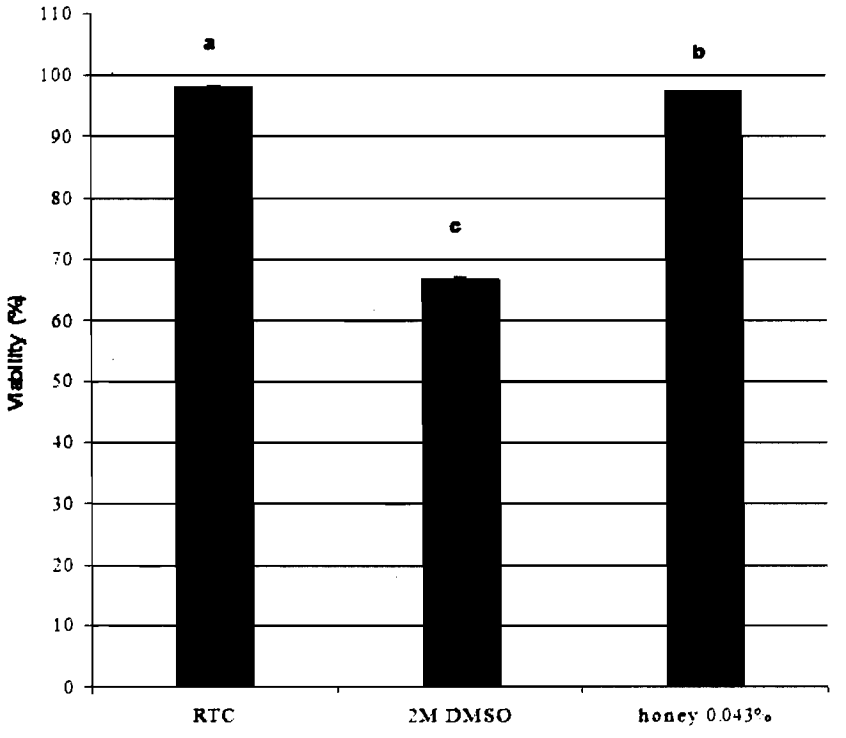

Fig 3.10 Blastomere survival after freeze-thawing and cryoprotectant removal. Blastomeres were incubated at room temperature for 60 min after cryoprotectant removal. Error bars represent standard errors. Significant differences are indicated in different letters ($n=3$, $p>0.05$).

Blastomere cryopreserved in 2M DMSO and 0.043% honey after 30 and 60 min incubation in PBS after freezing-thawing and CPT removal are shown in Fig 3.11. 30 min after cryoprotectant removal, 2M DMSO (89.5%) showed a significantly lower survival percentage when compared with those cryopreserved in 0.043% honey ($p> 0.05$). After 60 min incubation in PBS, blastomere survival cryopreserved in 2M DMSO, decreased to 66.8% whilst 97.2% survival was obtained from blastomeres cryopreserved in honey.

In this present study 0.043% honey was more effective than 2M DMSO in protecting blastomeres from freezing damage when compared with RTC (98.6%). The mean number of cells per 1.5 ml in each sample was 936 (range 864-1010).

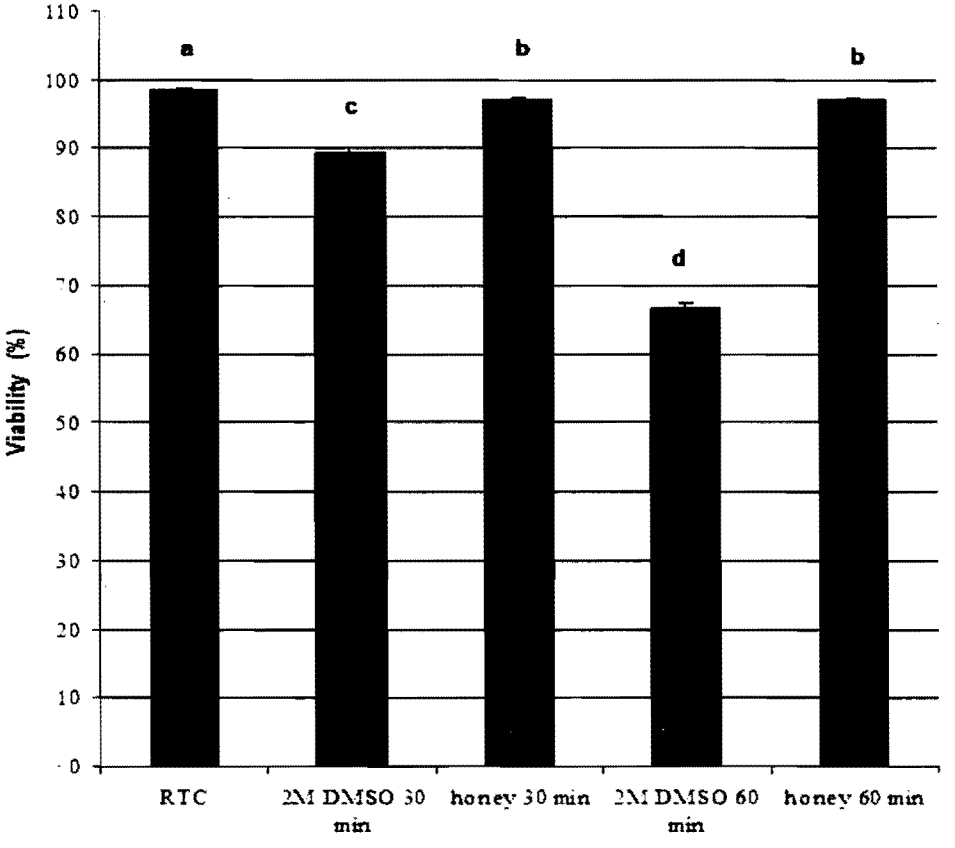

Fig 3.11 Comparisons of blastomere survival after 30 min and 60 min incubation in PBS at room temperature. Blastomeres were cryopreserved in 2M DMSO or 0.043% honey. Error bars represent standard errors. Significant differences are indicated in different letters (n=3, $p>0.05$).

3.3 DISCUSSIONS

3.3.1 STUDIES ON TOXICITY OF CRYOPROTECTANTS

There have been no published studies on toxicity of cryoprotectant to zebrafish blastomeres at 75% epiboly stage. However, studies on cryoprotectant toxicity to zebrafish embryos showed that methanol was the least toxic agent (Zhang et al., 1993). In addition numerous studies have suggested that methanol is the only cryoprotectant to permeate zebrafish embryos quickly (Zhang and Rawson, 1996; Hagedorn et al., 1997a). Methanol was therefore chosen as one of the cryoprotectants in this study for zebrafish blastomeres at 75% epiboly stage. DMSO has been the most commonly used cryoprotectant in fish sperm cryopreservation (Billard, 2001) and is also effective in cryopreservation of fish blood cells (Zhang et al., 1996) and fish embryonic cells (Leveroni et al., 1999). The common cryoprotectants ethylene glycol (EG) and propylene glycol (PG) have been recently studied for their toxicity to zebrafish oocytes (Plachinta et al., 2004). Results showed that toxicity of tested cryoprotectants appeared to increase with development stage with stage V oocytes being the most sensitive. No Observed Effect Concentrations (NOECs) for stage III oocytes were 2M for PG and 1M for EG. Previous findings on toxicity of CPAs to zebrafish embryos which also showed the relatively low toxicity of methanol and PG when compared with DMSO or EG (Zhang et al., 1996; Zhang et al., 1993).

Study of fish embryos has shown a relationship between embryo's chilling sensitivity and its development stage, including: brown trout (Maddock, 1974), rainbow trout (Haga, 1982) carp (Dinnyes et al. 1998; Jaoul and Roubard, 1982; Roubaud et al. 1985), fathead minnows (Cloud et al., 1988), goldfish (Liu et al., 1993), and zebrafish (Zhang and Rawson, 1995). According to Zhang (2004), most of the studies above show that beyond the 50% epiboly stage the embryos show a reduced sensitivity to chilling; however, it may also accelerate rapidly at temperatures below zero. The exact reasons for the stage-dependant chilling sensitivity of embryos is still not completely clear, nevertheless, research suggests that it may be linked to features such as the cell size, surface area, changes in the cell and tissue, number of cells, effectiveness of repair mechanism, enzymatic reactions, and volume ratios (Zhang, 2004). Although the use of cryoprotectants reduces the chilling injury damage at

subzero temperatures, the embryos are still sensible to damage. It has been hypothesized by Mazur et al. (1992b) that the chilling-sensitivity may also be due to a reduction in synchrony of reactions during the development of the embryo, though, this are requires further investigation.

Cryopreservation of zebrafish blastomeres has not been studied systematically so there is not enough information about cryoprotectant toxicity on zebrafish blastomeres, however, other marine species have been studied such as whiting (*Sillago japonica*); estuarine pejerrey (*Odontesthes bonariensis*); and freshwater medaka (*Oryzias latipes*). Cryopreservation of isolated fish blastomeres in three fish species representative of distinct environments was examined in order to study the toxicity of the cryoprotectant DMSO (dimethyl sulphoxide). Results of these three marine species showed that their blastomeres can tolerate DMSO concentrations to up to 9% relatively well for over 5 hs but lost viability rapidly at 18% (Strussmann et al., 1999).

Results from the present study on cryoprotectants toxicity to blastomeres from 75% epiboly stage showed that 2M DMSO and 2M methanol were less toxic than propylene glycol and ethylene glycol. DMSO and methanol were therefore used in controlled slow cooling experiments.

3.3.2 CRYOPRESERVATION OF 75% EPIBOLY STAGE BLASTOMERES USING CONTROLLED SLOW COOLING

When controlled slow cooling is used for cryopreservation of biological materials, it is important that cooling rate should be slow enough to permit adequate degree of cell dehydration during freezing which in combination with the presence of cryoprotectants prevents intracellular ice formation. On the other hand cooling rate needs to be fast enough to avoid overexposure of cells concentrated salt solutions. In this present study, slow cooling rate used of 1°C/min was used for blastomeres at 75% epiboly stage as it was reported to be optimum for rainbow trout at 6C stage (Leveroni and Maisse, 1998, 1999). Where as high as 97.2% blastomere survival was obtained.

Initial results with blastomeres at 75% epiboly stage after freezing using 2M DMSO and 2M methanol showed 2M DMSO was more effective than methanol in controlled slow cooling experiment. In the following experiments 2M DMSO was used, together with new compounds coffee, $NaHCO_3$ and honey.

Recent report from LIRANS on cryopreservation of zebrafish blastomeres at 50% epiboly using controlled slow cooling (Lin et al., 2007) also showed that DMSO was the most effective cryoprotectant when compared with EG, PG, and methanol. The highest blastomere survival of 63% was obtained with 1.5M DMSO after cryopreservation using controlled slow cooling at 0.3°C /min (after seeding from -7.5°C to -40°C).

Comparisons of the best results 97.2% obtained from the present study for 75% epiboly blastomeres with the above mentioned work indicated that blastomeres at 75% epiboly stage survived better after cryopreservation than the results obtained from Lin et al., (2007) for 50% epiboly blastomeres (63% survival).

It has been reported that earlier stage zebrafish embryos are much more sensitive to cryoprotectant toxicity than later stages (Zhang et al., 1993). Zebrafish embryos were also reported to be very chilling sensitive and the early development stage (<10 h) were the most sensitive to chilling injury (Zhang, 1994). Later stage embryos are much more resistant to cryoprotectant toxicity and chilling injury, this may explain why 75% epiboly stage blastomeres survived better after cryopreservation.

Blastomeres at 50% and 75% epiboly stages are of similar size (0.045 ± 0.0026 and 0.043 ± 0.0021mm) and should have similar surface to volumes ratios. Therefore, the differences between their survivals after cryopreservation cannot be explained by their size. More research is needed in this area.

Results obtained from blastomeres cryopreserved in 0.1% coffee, 0.0006% $NaHCO_3$ and 0.043 w/v % honey (Fig 3.8) showed that, blastomere survival was significantly higher in honey than in the other three compounds. Blastomeres cryopreserved in 0.043% honey showed 98.1% survival after 60 min incubation in PBS at room temperature following freeze-thawing and cryoprotectant removal.

CHAPTER 4

CONCLUSION

4.1 SUMMARY OF RESULTS

Toxicity studies of four cryoprotectants were carried out: DMSO (dimethyl sulphoxide), methanol, propylene glycol and ethylene glycol toxicity to 75% epiboly stage blastomeres were compared with room temperature controls (in PBS). DMSO and methanol were the least toxic cryoprotectants when compared with propylene glycol and ethylene glycol.

The No Observed Effect Concentration (NOEC) of DMSO, methanol propylene glycol and ethylene glycol were 2, 2, 1 and 1M. DMSO and methanol were therefore used in the subsequent experiments.

The following cryopreservation protocol for zebrafish blastomeres was used: 2°C/ min from 20 °C to -7.5 °C, after seeding, from -7.5°C to -40°C at 1°C /min; from -40 °C to -80°C at 5°C /min, samples were then plunged into liquid nitrogen.

Three additional compounds were also used as cryoprotectants: 0.1 % coffee, 0.0006% $NaHCO_3$, and 0.043 % honey, blastomeres survivals were assessed immediately after freezing with no cryoprotectant removal, and also after up to 60 min incubation in PBS at room temperature.

Results of these experiments showed that 0.043 w/v % honey provided the best survival rate (98.1%). However, survival of blastomeres cryopreserved in 2M DMSO (97.8%), 0.0006% $NaHCO_3$, (72.9%), 0.1% coffee (52.9%) were significantly decreased when compared with room temperature controls ($p<0.05$).

Effect of extended incubation period at room temperature on blastomeres survival after freezing-thawing and cryoprotectant removal was carried out with 2M DMSO and 0.043 % honey; blastomeres treated with 2M DMSO and 0.043 % honey were incubated at room temperature for 30 min and 60 min after cryoprotectant removal.

Results showed there were significant differences between room temperature control (98.6%) and 0.043 % honey (97.2%) or 2M DMSO (89.5%) blastomere survivals ($p < 0.05$) when compared with room temperature control (98.6%) after 30 min incubation, although blastomere survival in honey was significantly higher than in DMSO.

Comparisons of blastomere survivals after 30 min and 60 min incubation in PBS following freezing-thawing and cryoprotectant removal showed that blastomere cryopreserved in 2M DMSO (66.8%) deteriorated significantly when compared with 30 min incubation (89.5%). However, after 60 min incubation 0.043 % honey did not decrease blastomeres survivals (97.2%) when compared with 30 min incubation.

4.2 CONCLUSIONS

The results from the present study showed that zebrafish blastomeres (at 75% epiboly stage) treated with 0.043w/v % honey after 60 min cryoprotectant removal provided high rate survival of 97.2% after cryopreservation. The effectiveness of honey as cryoprotectant may due to its high sugar content. Sugars, such as glucose have proven to have membrane-stabilizing effect during cryopreservation and play an important role in preventing changes to cellular membrane during dehydration. The cryoprotective efficiency of glucose, sucrose or raffinose may be attributed to an activity dependent alteration, rather than specific solute-membrane interactions in the freezing environment (Lineberger and Steponkus, 1980).

Sugars such as sucrose and trehalose have also proven to inhibit the membrane mixing associated with chilling. Both sugars fit well in cell membranes, binding to phospholipid head groups.

The honey Formulation used contains a small amount of *Spirulina platensis*, which is particularly rich in protein and also contains carotenoids, minerals, and essential fatty acids. Peripheral http://en.wikipedia.org/wiki/Peripheral_membrane_protein membrane proteins are temporarily attached either to the http://en.wikipedia.org/wiki/Lipid_bilayer lipid bylayer or to integral proteins by a combination of hydophobic, electrostatic, and other non-covalent interactions, further

studies in spirulina protein effect may be considered useful in order to determine the relationship with the bilayer (Protein-lipid interactions).

This is the first time this product is used as cryoprotectant in zebrafish blastomeres cryopreservation and the comparisons were made with DMSO.

4.3 FUTURE WORK

More studies are needed to investigate the mechanisms of how honey can protect cells from freezing injury. Previous work has shown that sucrose and trehalose can protect cell membranes from freezing injury but the mechanisms of their action have remained unclear. As honey contains fructose, glucose and sugars, more investigations will be needed. Honey could be an important CPA for different type of cells so comparing the effectiveness of natural honey with other sugar mixtures will help to determine whether the degree of effectiveness of the CPA properties of honey is simply due to its sugar content or if the precise mixture of sugars as they occur naturally in honey is what produces the positive outcome.

Further research and cryopreservation experiments including honey as novel additive with blastomeres at all different stages from cleavage period (4-6 cells), Blastula period (128 cells-50% epiboly), Gastrula period (50% - 100% epiboly) will be necessary in order to evaluate the effectiveness of honey. Experiments on using honey for cryopreservation of early stage embryos and oocytes will also be an interesting challenge.

If honey can be established as an effective alternative cryoprotectant, it would be important to consider running freezing protocols with some of the many different types of blossom as they can vary significantly on their chemical and therefore cryoprotective properties.

Investigations on survival of honey protected blastomeres in culture post-thaw would be essential as the cell viability asessement method used in the present study (trypan blue staining) only assess the membrane integrity of the cells and not their development potentials.

Earlier work has shown that sucrose and trehalose protect membranes and proteins with high viability results in bacterias during drying; nevertheless the mechanism of their action has remained unclear (Leslie et al., 1995).

The extracellular, non-permeating cryoprotectants studies as sugars have various advantageous effects on the vitrification process develop new methods to utilize honey as a novel additive will be valuable.

Studies on culture of cryopreserved blastomeres for longer period (weeks) should also be carried out to investigate the long-term survival.

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